Social stress as a source of reduced welfare in pigs

Ruis, Markus Adrianus Wilhelmus

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Promotor:	Prof. dr. J.M. Koolhaas
Referent:	Dr. ir. H.J. Blokhuis
Beoordelingscommissie:	Prof. dr. P. Jensen Prof. dr. B.M. Spruijt Prof. dr. A.B. Steffens

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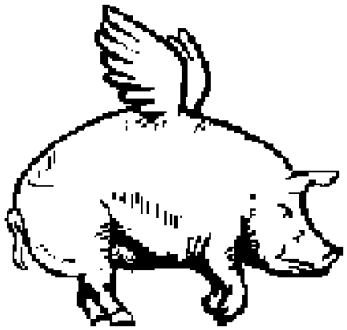
If you didn't care what happened to me,
And I didn't care for you,
We would zig zag our way through the boredom and pain,
Occasionally glancing up through the rain,
Wondering which of the buggers to blame
And watching for pigs on the wing.

Pigs on the Wing (Part One)
Pink Floyd - Animals



Aan Leonie, Silvio, .., en mijn moeder
Ter nagedachtenis aan mijn vader

The research described in this thesis was carried out at the Department of Behaviour, Stress Physiology and Management, Institute for Animal Science and Health (ID-Lelystad), Lelystad, The Netherlands, and at the Department of Animal Physiology, Graduate School for Behavioral and Cognitive Neurosciences (BCN), University of Groningen, The Netherlands. The research was funded by the Dutch Organization for Scientific Research (NWO; ALW-research programme 'Determinants of adaptive capacity') and the Dutch Ministry of Agriculture, Nature Management and Fisheries. The publication of this thesis was financially supported by ID-Lelystad, the University of Groningen, and the Research Institute for Animal Husbandry (Praktijkonderzoek Veehouderij (PV)), Lelystad, The Netherlands.

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Contents

Chapter 1

General introduction

Animal welfare

In commercial farming, human drive for higher production, economic benefits, labour efficiency and reduction of environmental emissions, led to the development of intensive husbandry techniques which are very demanding for the animals and cause reduced welfare. Over the last decades, however, interest in and concern about animal welfare has been increasing in many countries. Consumers more and more require 'welfare-friendly' products, and show growing interest in organic food products. This is increasingly supported by legislation aiming to improve farm animal welfare. In The Netherlands, the growing public concern for animal welfare led to the establishment of the Dutch Animal Health and Welfare Act (Gezondheids- en Welzijnswet voor Dieren, 1992). However, there is still much debate on the issue of animal welfare. Different parties, such as farmers, welfare organisations, consumers and policy makers, often have different opinions, perceptions, views and values, regarding what specifically constitutes animal welfare. Scientists have an important task to select objective variables to provide information on actual, rather than perceived, welfare. The problem is that animal welfare is a concept that can neither be viewed in a purely objective manner, nor simply defined, described or assessed. It is not a scientifically or technically precise state, but rather a multidimensional one (Clark, 1997a). Most definitions agree that welfare involves the quality of life and concerns the relationship between animals and their environment. Welfare of an animal has often been characterized as a state of mental and physical health when living in harmony with the environment (Duncan and Dawkins, 1983). Wiepkema and Koolhaas (1993) have added a biological translation: 'Welfare is present when an individual can reliably predict or control relevant events by means of species-specific signals and means'. One of the most frequently used definitions of animal welfare was stated by Broom and Johnson (1993): 'the welfare of an individual is its state as regards its attempts to cope with its environment'. The 'state as regards attempts to cope' refers to how much has to be done in order to adapt to the environment. The more effort has to be put into adaptation, the poorer animal welfare. Especially when controllability of the environment becomes low, an animal may have to put much (mental) effort into its adaptation, or its adaptation may even fail. This may lead to a state of stress. Duncan (1996) stated 'it is the negative emotions of feeling stressed or frightened or in pain that reduce welfare'. In the following section, a definition of stress is given and the implications of (chronic) stress for welfare are being discussed. Moreover, several indicators (measures) of welfare are being described.

Measurements of welfare

The extent to what is done to adapt or to cope, and effects of a lack of adaptation, can be measured in terms of behaviour, physiology, health and production. In the following I discuss the assessment of welfare in terms of measurements of stress and (limits of) adaptation, production and health.

Stress and adaptation

The concept of stress is an integral part of discussions on welfare. The term stress is often used to indicate a physical or mental burden on the animal, indicative for reduced welfare. This view, however, is too simple. This is mainly attributed to the variety of definitions of stress, depending on the area of work they refer to, and both describing cause and effect. I will use the term stress to indicate an animal's internal state (effect), and the term stressor to indicate the source of stress (cause). As most generally used now, stress refers to a state of threatened homeostasis (body equilibrium; Clark, 1997b). To counteract disturbances in homeostasis, the body reacts with stress responses, serving a regulatory (adaptive) function. In the early studies on stress, it was thought that stressors were mainly of physical nature, and that stress responses were non-specific (Selye, 1935; 1946). Later studies revealed that psychological components of stressors were the more important determinants of the stress response, and that different stressors evoke their own and often specific stress responses (Mason, 1971; Levine et al., 1989). According to Weiss (1968), the perception of the stimulus and consequently the degree of control is the responsible variable in determining the stress response.

Stress responses are thus normally adaptive when the animal is able to cope with the challenge and satisfactorily handles the threat. Some environmental instability and uncertainty, leading to stress responses, may even be beneficial to avoid boredom and optimize individual vigilance (Wemelsfelder, 1990; Wiepkema and Koolhaas, 1993). Adaptation occurs by activation of a complex pattern of species-specific behavioural changes, and responses of the central and autonomic nervous and endocrine systems:

Behavioural responses. When environmental conditions change, animals often respond with changes in behaviour. Behavioural changes are among the most easily observed indicators for a state of stress. However, for proper interpretations of behavioural responses or changes in the quality of behaviours, in terms of stress, knowledge of species-specific behaviour is required. The *delay* or *latency* of resuming normal behaviour following an environmental change can be a useful measure of stress (Broom and Johnson, 1993). Situations of uncertainty and threat

may lead to behavioural changes, which include those of agonistic behaviour, displacement or interruptive behaviour, and redirected behaviour (Wiepkema and Koolhaas, 1993). These behaviours are generally short-lived and are part of normal regulatory mechanisms to solve the conflict.

Physiological responses. Physiological adaptation to stressors consists of activation of several physiological systems, including the hypothalamic-pituitary-adrenal (HPA) axis (Harbuz and Lightman, 1992; Minton, 1994; Selye, 1935), the autonomic nervous system (Axelrod and Reisine, 1984; Cannon, 1914), and the immune system (Harbuz and Lightman, 1992). The magnitude of physiological responses may represent the adversity of the threat and consequently the degree of adaptation. Secretion of *ACTH* (from the pituitary) and *corticosteroids* (from the adrenal cortex) are the final steps in the activation of the *HPA-axis*. HPA responses serve to restore homeostasis by their effects on metabolism. More frequently than ACTH, corticosteroids are used as important indicators of stress. However, there are some limitations. For instance, changes in the HPA-axis are not always accompanied by changes in corticosteroids (Janssens et al., 1995b; Ladewig and Smidt, 1989). Also, there are stressful conditions to which adrenals do not respond (Moberg, 1985). Simultaneous measurements of ACTH and corticosteroid concentrations may therefore provide better information on HPA-axis activity. Most studies measure the plasma total (free and bound) corticosteroid concentration, rather than the free fraction. The latter fraction is in the biologically active form, and can be measured in other body fluids, such as saliva (Vining et al., 1983). The technique of collecting saliva is often more simple and much less invasive than that of blood sampling (Parrott et al., 1989). The activity of the *autonomic nervous system* is determined by its actions of the sympathetic and parasympathetic branches. The sympathetic action results in a very fast (within seconds) release of *catecholamines*: noradrenaline from the sympathetic nerve endings and adrenaline from the adrenal medulla. This action leads to increases in heart rate, enabling the animal to make a quick physiological adjustment to a sudden threat. Relatively few studies have characterized catecholamine responses to stressors in farm animals. This is undoubtedly due to the difficulties in rapid enough sampling of blood, requiring catherizations of animals. The latter may become problematic when animals are housed in groups, and group-mates are able to destroy each others catheters. Sampling of urine for catecholamine measurements is a promising alternative (Hay et al., 2000). *Heart rate* is often measured in response to stressors, but it is difficult to separate physical and psychological causes. Assessment *of heart rate variability*, in addition to heart rate,

may also include the parasympathetic action in the interpretation of stress levels. The parasympathetic and sympathetic branches work in opposite directions, and their balance determines heart rate frequency. Stress may alter this balance between the sympathetic and parasympathetic branches. Generally a higher heart rate variability represents a shift towards parasympathetic prevalence and a lower heart rate variability represents a predominant sympathetic action (Sgoifo et al., 1999). Alterations in the autonomic balance may thus indicate a change in the internal state of animals (Hansen and Von Borell, 1998; Forde and Marchant, 1999, De Jong, 2000), but the type of stressor determines the direction of the shift (Sgoifo et al., 1999). Other hormones involved in stress responses, but which are only mentioned here, are beta-endorphin, prolactin, vasopressin and glucagon (Sapolski, 1992). To conclude, the adaptive role of physiological mechanisms is also emphasized by effects of stress on *cognitive function*. Moderate and short-term increases in catecholamines and cortisol can enhance memory, learning and cognition processes (Mendl 1999). This may improve the animals efficiency of reactions and adaptation when repeatedly encountering potential threats.

Immune responses. The immune system plays an important role in the resistance to diseases. Stress-induced changes in immune activity are complex and widely affected by neuroendocrine parameters and neurotransmitters. Conversely, humoral components of the immune response exert effects on the central and peripheral components of stress responses (Kelley, 1988). In response to a stressor, some immune responses are enhanced whereas others are suppressed. Generally, acute stress enhances aspecific reactions, and increases are found in numbers of blood phagocytic cells (Wallgren et al., 1994) and phagocytic activity (Ruis and Bayne, 1997). These reactions normally serve as a primary defence against invading pathogens. Specific responses, such as lymphocyte proliferation and antibody production are a second line of defence and are initially suppressed. This suppression may serve to protect the body against an over-activation of defense mechanisms (Munck et al., 1984). Accordingly, for assessments of stress, measurements of changes in leucocyte subsets in blood may provide a valuable and easily applicable tool (Wallgren et al., 1994). Catecholamines seem to be the main mediators in the enhancement of aspects of the immune response (Croiset et al., 1987), whereas corticosteroids have immunosuppressive and anti-inflammatory effects (Munck et al., 1984; Wallgren et al., 1994).

Stress and adaptive capacity

Stress may become a burden to an animal when stressors have a long lasting (after-)effect. When stressors are mild and short-lived, animal welfare is normally not at risk. Stress responses, however, may be extended or unsuccesful, and an animal may have to put much effort into its adaptation or is not able to adapt, leading to (pre)pathological states. For many farm animals, threatening situations are often beyond their control and adaptive capacity. These situations may involve the permanent presence of a stressor, for instance, when the internal need to perform normal species-specific behaviour cannot be fulfilled, but may also result from the occurrence of one or few experiences with severe stressors (major life events) (Wiepkema and Koolhaas, 1993). When the stress response does not lead to a desired adaptation, behavioural symptoms of chronic stress may develop. These include *abnormal behaviours* such as violent aggression in frustrating situations, disturbed behaviours such as injurious and stereotypic behaviour, and apathy and unresponsiveness (Broom and Johnson, 1993; Wiepkema and Koolhaas, 1993). A persistent threat may also lead to prolonged *hyperactivity of physiological systems*, which impairs rather than contributes to well-being. Under such chronic stress conditions, changes in baseline activities of physiological systems may increase the occurrence of certain stress pathologies. Long-term sympathetic activation, for instance, may enhance the risk for hypertension and artherosclerosis, whereas prolonged parasympathetic activation may lead to heart rhythm disturbances and sudden cardiac death (Koolhaas et al., 1999; Sgoifo et al., 1999). Chronically increased corticosteroid levels may increase the formation of *gastro-intestinal ulcers*, reduce the size of various *organs* such as the thymus, spleen and reproductive organs (Selye, 1946), cause *neuronal damage* (Sapolsky, 1996), and enhance the risks of infection due to a permanent *immunosuppression* (Kelley, 1988; Munck et al., 1984; Wallgren et al., 1994). Chronic stress may also lead to long-term changes in *body temperature*, but the direction (hyper- or hypothermia) and duration depends on the type of stressor (Broom and Johnson, 1993; De Jong, 2000).

Signs of stress pathologies may also develop while no changes in baseline activity of physiological parameters are detected. In these situations, the regulatory feedback mechanisms may restore baseline levels, although normal functioning of physiological systems is altered for a prolonged time (Wiepkema and Koolhaas, 1993). Such an alteration may be observed following a *major life event*. Following such an acute but very severe stressor, changes in baseline levels may not be detected, whereas *reactivity of physiological systems* may be altered for a

prolonged time (Koolhaas et al., 1997b). For the HPA-axis, tests of feedback mechanisms (by dexamethasone) and stimulation (by CRF, ACTH or mild stressors) are best suited to study the functional state of the system (Mormède et al., 1984). The physiological state, together with behavioural characteristics, may also say something about the *emotional state or mood* of an animal. Although mood disorders in animals cannot directly be assessed and we cannot be certain what an animals feelings are, a certain set of behavioural and physiological reactions may point to the existence of stress disorders which resemble those in humans. For instance, in animals, observed reductions in mobility and activity (Koolhaas et al., 1990; Meerlo et al., 1996b) and hyperactivity of the HPA-axis (Buwalda et al., 1999), much resemble symptoms of human *depression*. The similarity with this human psychopathology was also demonstrated by the succesful counteraction of mentioned behavioural changes, with pharmaca normally used to treat human depressive states (Koolhaas et al., 1990). *Fear* is another aversive emotional state, following relative complex cognitive processes, which normally serves to avoid potentially harmful situations (Boissy, 1995; Rushen et al., 1999a). Fear, however, may become less functional when animals are not able to distinguish between threatening and non-threatening stimuli, or when an animal reacts more and more aversively, even though the (minor) stimulus is rather constant.

Mood disorders may thus lead to increases in responses to continuing or repeated stimulation. In other words, the animal becomes more vulnerable to changes in the environment. This process is called *sensitization* (Koolhaas et al., 1997b; Post, 1992). This contrasts the process of *habituation*, in which animals reduce their responses to repeated stimuli. This waning of stress responses is considered normally adaptive, and is a process of learning (Broom and Johnson, 1993). In contrast to moderate corticosteroid responses (discussed earlier), excessively high or prolonged elevations of corticosteroids can lead to impairment of cognitive performance and memory function (Mendl, 1999). A common element in fear-evoking situations is novelty. Therefore, indications of the emotional status of animals are often tested by introduction of stimuli which include novelty. Stress responses to these tests, such as explorative and locomotory behaviours, and physiological reactions, are expected to be higher when animals are generally more fearful. Not until recently, some of the standard tests were pharmacologically (with anxiolytica) validated to be useful for measurements of fear in farm animals (Andersen et al., 2000b; Hopster et al., 1999).

Chronic stress, either by persistence of the stressor or single experiences with severe stressors (major life events), thus seems to constitute the greatest risk for animal welfare. Abnormal behaviours, disruptions in physiological systems, decreased immune capacity and mood disorders, are reliable and objective indicators of a state of reduced welfare. However, especially during prepathological states, it is not always clear whether the stress response is adaptive or not, and whether a situation of chronic stress exists. In other words, it is frequently unclear when the border is crossed between a functional adaptive response and a response that is not adaptive. For a better interpretation, in terms of welfare, it is always important to focus simultaneously on different aspects of the stress response, i.e. on characteristics of behaviour, physiology and emotionality.

Production

Production can cover a great variety of entities, but *body weight* or *body growth* is most often referred to. Body growth, which is relatively easy to measure, may indicate whether some important requirements with respect to welfare are met. Impaired body growth following a period of normal growth may be an indication of reduced welfare (Broom and Johnson, 1993; Wiepkema and Koolhaas, 1993). Good production results, on the other hand, are not necessarily proof of optimal welfare. For instance, poor welfare is expected in fast growing broilers that are physiologically unable to manage with this growth. Furthermore, animals may meet higher energy demands without effects on body growth. This may happen in situations of higher activity, but also under conditions of stress (Stookey and Gonyou, 1994). *Feed efficiency*, as the ratio between body weight gain and feed intake (gain/feed), may therefore give extra information on the metabolic state of an animal.

Health

Health, or freedom from disease and physical damage (injuries), is a very important aspect in the assessments of animal welfare. Decreased health may be directly related to chronic stress: e.g. a higher vulnerability to disease (pathology) and aggression-related skin damage. A way to assess vulnerability to diseases is to test immune system function, by deliberately infecting animals with pathogens and determine specific responses of the immune system. One refinement is the use of vaccination-challenge models, giving information about the immune response (lymphocyte proliferation, antibody production, cytokine production), the memory response and symptoms of clinical illness (fever response, virus-excretion). Other

factors which are also known to have an important effect on the health status of an animal include the presence of pathogens, accidents related to poor housing design and poor management (i.e. slippery floors), and human interventions to prevent behavioural problems resulting from intensive farming (i.e beaktrimming of laying hens, teeth and tail clipping of piglets, dehorning of dairy cattle). Physical damage as such may be very painful, which is a very aversive sensation for the animal. Pain therefore constitutes a primary aspect of welfare and pain reactions may be recognized by behavioural and physiological changes and responses (Broom and Johnson, 1993).

Welfare of growing pigs: the subject of this thesis

The development of intensive husbandry systems took also place in commercial farming of pigs. The domesticated pig is now one of the most intensively kept farm animals in The Netherlands. In 1996, the human population in The Netherlands was even almost outnumbered by the number of pigs. This high pig production is accompanied with a physical and social living environment which is very limited and includes many potential hazards. Gestating and farrowing sows are often placed in close confinement stalls, and growing pigs are housed in fully or partly slatted systems, with little space and no substrate. Importantly, social conditions resulting from housing and management routines in agricultural systems are often far from desired in view of the biology of the pig (see next section). This may lead to situations that an animal has to put much (mental) effort into its adaptation or its control of the (social) environment. Consequently, this may lead to a state of (chronic) stress. Because social conditions are the source of this stress (social stressor), this type of stress is also referred to as social stress.

Pigs may experience high levels of social stress when socially mixed with unfamiliar (strange) conspecifics, which is generally recognized as a major welfare concern in intensive pig farming. Mixing usually induces vigorous fighting and aggression to settle social hierarchy positions (see also next section). The aim of the project described in this thesis was to investigate which social component(s) is (are) responsible for or modulate the adverse effects of mixing: i.e. initial fighting to establish a social hierarchy, prolonged coexistence of unfamiliar pigs, social support, and individual characteristics (coping characteristics). Moreover, by investigating effects of social isolation, the importance of having social contact with conspecifics as such was determined. Because of the nature of the welfare concept, assessments of welfare status were based on multifacetted approaches, by an integration of measurements of stress responses, indications of emotional status,

and production and health variables (Figure 1). Despite the fundamental approach of this project, it was also aimed to develop or optimize tools with practical value for pig husbandry, leading to improved husbandry methods.

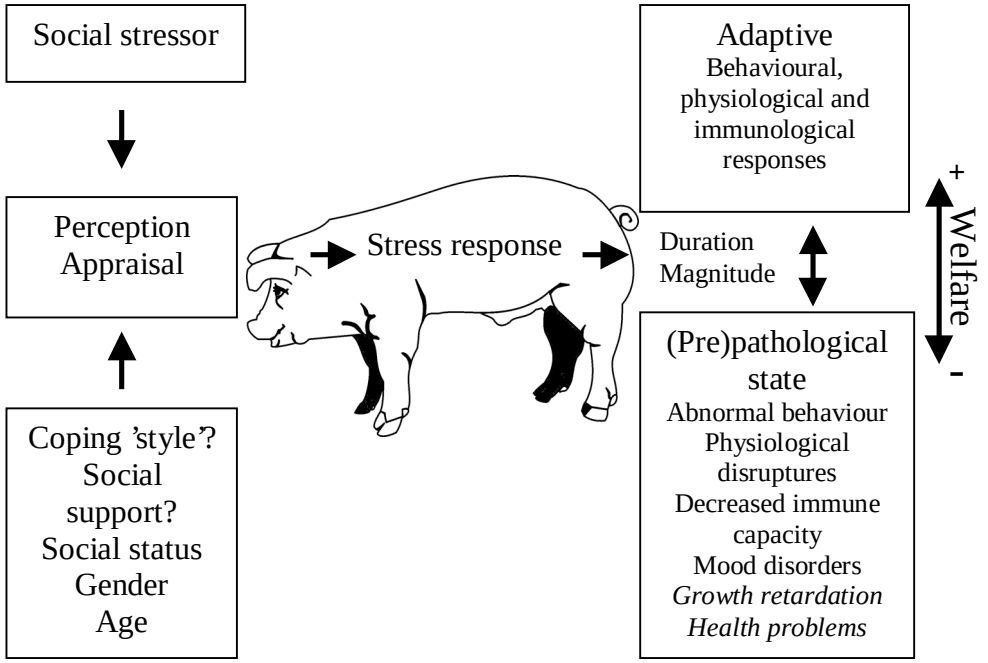

Figure 1. Schematic overview of the potential stress response to a social stressor, and its implications for pig welfare. Modulating effects of factors with questionmarks are studied in the current project.

Pig-specific behaviour: social relationships

The domesticated pig is generally accepted to originate from wild pigs of the species *Sus scrofa* which ranges throughout the woodlands of Europe, North Africa and Asia. Wild pigs are highly social animals and live in groups of two to five related females and their immature offspring (a number of unweaned juveniles and weaned sub-adults). From 1-1.5 years of age, boars start to leave these social groups and tend to remain solitary. They will temporarily join social groups during the mating season. In general, the organization of a social group is based upon a dominance hierarchy, which gradually develops while animals grow up. Piglets already start to form social dominance relationships within hours after birth (Graves, 1984). As a consequence, the members of a social system are characterized by differences in social status, with dominants having priority of access to resources.

Despite the long history of domestication, estimated to have begun around 7000 years B.C., the domesticated pig still shows behaviour that includes most of that of their wild ancestors (Jensen, 1986; Stolba and Wood-Gush, 1984). This was shown by studying domesticated pigs in semi-natural settings. These studies also revealed that familiar pigs tend to form subgroups, whereas the unfamiliar pigs maintain separate sleeping areas for a prolonged period (over six months). Generally, familiar animals show social bonding, and the strongest social relationships exist in littermates (Newberry and Wood-Gush, 1986; Petersen et al., 1989). Unfamiliarity, on the other hand, often leads to much fighting and aggression (agonistic behaviour), which may serve to drive away a stranger (Puppe, 1998). However, when animals become part of the same social group, agonistic behaviour may also lead to and maintain social dominance relationships. (Meese and Ewbank, 1973).

When pigs are housed in intensive agricultural systems, groupings imposed on pigs are not those which would apply in the wild. Growing pigs in modern production systems are mixed and kept in groups of unfamiliar and similar sized conspecifics. Both factors are known to increase the likelyhood and severity of fighting and aggression (McGlone and Curtis, 1985; Rushen, 1987). Moreover, after establishment of the social hierarchy, which cannot develop as gradually as compared to the situation in the wild, less intense aggression may be observed in the longer term (Ekkel et al., 1997; Stookey and Gonyou, 1994). This may be due to small living areas with no hiding places, leading to social friction and stress: animals cannot be expelled from the social group, some pigs are not able to settle dominance relationships, and dominant and subordinate animals sense each other's presence continuously. Often, limited access to food repeatedly triggers agonistic interactions. Health problems and decreased body growth are very common following mixing procedures (Ekkel et al., 1995b; Stookey and Gonyou, 1994). Generally, dominant pigs have more control over the situation, because they have preferred access to resources such as food (McGlone, 1986). Therefore, subordinate pigs are often assumed to be worst off in these artificial social groups. Dominant animals, on the other hand, may pay a price as well, for maintaining their high social status (threat to control).

Individual differences

Usually, a considerable variation between stress responses of different animals within the same species is observed. Based on clinical observations and literature studies, Engel (1967) suggested the existence of basically two modes of

stress responses. One was referred to as the *'fight/flight'* response and was originally described by Cannon (1914). This response is aimed at the removal from the stressor, and is preferentially accompanied with an activation of the sympathetic nervous system. The second type of stress response was called the *'conservation/withdrawal'* response, and serves to reduce the emotional impact of the stressor. In Engel's view, this mode of stress response is characterized by immobility, and activation of the HPA-axis. Henry and Stephens (1977) supported this hypothesis of the existence of different stress response patterns, but they considered the conservation-withdrawal response as a form of pathology induced by a loss of control. This was opposed by Koolhaas et al. (1986), who suggested that the variation in stress responses reflect biologically functional variation in ways of coping or adaptation. They introduced the concept of *coping styles*, and introduced the terms *'active' and 'passive' coping*, which refer to the fight/flight and conservation-withdrawal responses, respectively. Both genetic factors and early life experiences were found to determine an animal's preferred way of coping with a variety of social and non-social stimuli (Benus et al., 1991). Perception or cognitive appraisal, and not the physical characteristics of a stressor per se, therefore determine the way of coping (Moberg, 1985; Koolhaas et al., 1999). Studies in rodents revealed that differentiation in levels of aggression constitutes another main characteristic of differences between ways of coping. Generally, aggressive animals show an active response to aversive situations, and low-aggressive individuals generally adopt a passive strategy when challenged (Benus et al., 1991; Bohus et al, 1987). In rat colonies, a relationship was found between coping characteristics and social dominance, with the dominant position usually being taken by an animal with an active strategy (Koolhaas and Bohus, 1989). Recently, it was proposed to replace the terms active and passive coping by new terms: *'proactive' and 'reactive' coping*, respectively. These new terms give a better description of the fundamental difference between the two styles (Koolhaas et al., 1997a; 1999), which is prominently based on the degree in which behaviour is guided by environmental stimuli. High-aggressive animals easily develop routines, i.e. their behaviour is intrinsically driven. They have a more proactive type of behavioural response. Low-aggressive animals seem to be more adaptive and flexible, and react to environmental stimuli all the time. Therefore, proactive and reactive animals may have advantages under stable and variable conditions, respectively (Benus et al., 1991; Koolhaas et al., 1997a; 1999).

In rodents, genetic selection may have exaggerated coping patterns to a level normally not found in a natural population. However, few studies in feral

populations, e.g. in the wild house mouse (Van Oortmerssen and Busser, 1989) and the great tit (Verbeek et al., 1994), indicated the existence of distinct phenotypes. A differentiation in coping styles may be highly functional in population dynamics, and natural selection will tend to retain both categories of animals. However, it is questionable whether under farming conditions, which comprise standardized housing and management routines, it is benefial to have different ways of coping. One might imagine that domestication may have affected the distribution of individual variation, which may have favoured one or another type of animal (Hopster, 1998; Jensen et al., 1995). It is also suggested that domestication is accompanied with changes in the quantitative rather than in the qualitative nature of responses (Price, 1999). Either way, the domesticated pig still shows considerable individual variability in response to environmental stimuli. Attempts to categorize pigs according to ways of coping were less succesful and showed divergent results. Some autors state that behavioural and physiological responses remain constant within individual pigs across situations (Hessing et al., 1994b; Mendl et al., 1992; Thodberg et al., 1999), which was opposed by others (Forkman et al., 1995; Jensen et al., 1995). Moreover, tested populations of pigs did not show non-normal distributions of their stress responses, as bi- or multi-modality should exist (Forkman et al., 1995; Jensen et al., 1995). However, the shape of the distribution curve does not seem to matter much when individual vulnerability to stress-related problems is concerned (Koolhaas et al., 1999). Recent studies in pigs provided some indications that extreme responders to two different restraint tests, e.g. the backtest (Hessing et al., 1994b) and the so called tonic immobility test (Erhard and Mendl, 1999; Erhard et al., 1999), show different levels of aggression and have different patterns of stress responses in various situations. It may therefore be speculated, when qualitative and quantitative differences between extremes are large enough, that specific pathologies develop in situations of chronic stress, related to specific physiological states. Individual pigs may then differ in susceptibility to show abnormal behaviour, and to develop cardiovascular disease, ulcer formation, stereotypies and infectious disease (Koolhaas et al., 1999). Furthermore, in groupings of pigs, behavioural characteristics of individual animals, such as individual aggressiveness, may have an important influence on the establishment of a stable social hierarchy (Erhard et al., 1997; Hessing et al., 1994a). Knowledge on individual ways of coping of farmed pigs is thus of utmost importance with regard to welfare.

The experiments

The purpose of this thesis was to investigate which social component(s) is (are) responsible for or modulate the adverse effects of mixing unfamiliar pigs: i.e. initial fighting to establish a social hierarchy, prolonged coexistence of unfamiliar pigs, social support, and individual characteristics (coping characteristics). Due to practical limitations, most of the experiments were restricted to experimental testings of young female pigs (gilts). Gilts were chosen because in barrows indications were obtained for long-term negative effects of castration (Chapters 2 and 5), which may confound with the outcome of experimental testings.

Chapter 2. In considering concentrations of corticosteroids as a physiological indicator of decreased welfare, knowledge on basal concentrations covering 24-hour periods is needed. Changes in corticosteroid concentrations may occur without the presence of stressors, due to fluctuations on a daily basis (circadian rhythmicity). Given stressors may also elicit different corticosteroid responses at different phases of the circadian cycle (Halberg, 1969; Klemcke et al., 1989). Furthermore, exposure to stressors may lead to disruptions of circadian rhythms, which may lead to reduced health and welfare (De Jong, 2000; Turek, 1994). Corticosteroid concentrations may also differ according to age (Evans et al., 1988) and gender (Nyberg et al., 1988). Therefore, the project was started with an investigation of the circadian rhythmicity of cortisol in saliva of growing pigs, in relation to age, gender and (time of) stressor application. We considered salivary cortisol as a reliable and convenient measure of stress for our project because it represents the biologically free fraction and saliva samplings in pigs are relatively easy and noninvasive (compared to blood samplings). During this experiment, a radioimmunoassay (RIA) for determinations of salivary cortisol concentrations was optimized and validated.

Chapter 3. It is not known whether adverse effects associated with regrouping or mixing is attributed to the initial fighting to establish a social hierarchy, to a continuing social stress by ongoing social conflicts between unfamiliar pigs in newly mixed groups, or by a combination of both. To elucidate this, it was first investigated what the behavioural and physiological consequences are of initial fighting for dominance, leading to dominant and subordinate (defeated) animals. It was expected that social confrontations are most stressful for loser animals, and therefore we started with studying the consequences of fighting for those animals being defeated. In male rats, a single social defeat may be considered as a major life event because of its long-term negative effects. Social defeat as a stressor is even used as a model for studies of human psychopathologies

(Koolhaas et al., 1997b). However, the social defeat model in rats was developed using rats that were individually housed. This type of housing is rather unnatural because wild rats live in groups. It is not known whether rats which are able to have contact with familiar conspecifics following social defeat would have similar long-lasting problems. It might be a general rule that (certain) familiar animals act as bonding (social) partners and provide social support which has positive consequences for individuals by reducing neuroendocrine changes in stressful situations (Henry and Stephens, 1977; Sachser et al., 1998). Therefore, we first wanted to study the validity of the defeat model in rats by modulating the social environment following defeat, i.e. individual versus group-housing. Observations were done during three weeks and included those of behavioural mobility and activity, anxiety, HPA-activity, body growth and size of various organs.

Chapter 4. As it turned out in Chapter 3, in rats the social environment following social defeat played an important modulating role in the dynamics of stress responses to the social stressor. Based on this information, acute and long-term effects of acute social defeat and modulating effects of the social environment were investigated in pairs of growing pigs. Only gilts were subject of study. An important difference with the previous experiment was that also non-defeated animals were part of study, which were either left undisturbed with their pen-mate or socially isolated. By studying socially isolated pigs, the importance of having contact with conspecifics as such was determined. Measurements included those of various behavioural, physiological and immunological changes, but also of emotionality and body growth.

Chapter 5. From Chapter 4 it was concluded that in pigs the often described negative effects of mixing are probably not only related to acute social defeat. In this experiment we therefore used mixing, i.e coexistence of unfamiliar pigs, as a stress paradigm which has the highest risk for welfare, and investigated its effect on behavioural, physiological and specific immune response parameters. Specific long-term immune responses and protection against challenge infection after vaccination with pseudorabies virus (PRV) were examined in Specific Pathogen Free pigs. These pigs were mixed pair-wise with an unfamiliar same-gender conspecific, or left undisturbed with a same-gender litter-mate. Effects of gender and social status were part of study.

Chapter 6. The aim of the experiment presented in this chapter was to determine whether the generally observed individual variation in behavioural and physiological (stress) responses of gilts to various challenges may point to fundamental differences in ways of coping. Consistency in reactions to novel

stimuli and in aggressive features were tested. Moreover, it was tried to replicate results of Hessing et al. (1993; 1994b) concerning classification of pigs into proactive and reactive responders according to the backtest. When pigs with different ways of coping exist, this characteristic of individual animals may be an important aspect in explaining (the stability of) relations among animals in social groups.

Chapter 7. As it turned out in the previous experiment that representatives of reactive and proactive pigs indeed can be found among farmed pigs, we accordingly designed an experiment to mix pigs (pair-wise) on the basis of their coping characteristics. By confronting unfamiliar gilts with either similar or different coping strategies it was tried to identify whether coping is a factor that explains social stress and risks for welfare following mixing. Implications for welfare were studied by observations of behaviour, physiology, emotionality, health and production. The relationship between social status and coping strategy was also investigated.

Chapter 8. Finally, to gain more insight in the success of respective coping strategies, a fundamental study was carried out to investigate differences between reactive and proactive gilts in their capacities to deal with a lack of any social contact with conspecifics, i.e. social isolation. On the basis of knowledge on coping strategies in rodents, individual pigs may differ in the perception and strategy to adapt to social isolation. For welfare assessments, similar variables as in Chapter 7 were used, extended with post-mortem observations.

Chapter 9. In this Chapter, the major findings of chapters 2-8 are summarized and discussed.

Chapter 2

The circadian rhythm of salivary cortisol in growing pigs: effects of age, gender and stress

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M.A.W. Ruis[1,2], J.H.A. te Brake[1], B. Engel[1], E.D. Ekkel[1], W.G. Buist[1], H.J. Blokhuis[1], J.M. Koolhaas[2]

[1] Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB Lelystad, The Netherlands
[2] Department of Animal Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

Abstract

This experiment was designed to examine circadian rhythmicity of cortisol in saliva of growing pigs, in relation to age, gender and (time of) stressor application. Additionally, the acute cortisol response to a stressor was studied. Five groups, each consisting of 3 barrows and 3 gilts, were involved in the experiment. In a control group, saliva samples were taken at 1-hour intervals at 12, 16, 20 and 24 weeks of age. Within one week, rhythmicity of cortisol was assessed during two 24-hour spans (Monday and Friday). Rhythm characteristics were evaluated by cosinor analysis, describing the rhythm by several parameters. In 2 groups at 12 weeks and 2 other groups at 20 weeks of age, a stressor was applied (4 hours of isolation) on Thursday morning or evening. Again, rhythmicity was assessed on Monday and Friday by sampling at 2-hour intervals. Acute cortisol effects were studied by sampling at several timepoints during isolation. Between 12 and 24 weeks of age, basal cortisol concentrations decreased and a rather stable and adult circadian rhythm was reached at 20 weeks of age. Average basal cortisol concentrations were higher in barrows than in gilts. Furthermore, after isolation, the amplitude of the rhythm was increased in barrows but was unchanged in gilts. The rhythm was more unstable and the maximum value tended to shift only after evening isolation. Stressor timing, but also age, was found to affect average cortisol concentrations. Moreover, stressor timing was important for the acute cortisol response: the increase was higher in the morning. The results of this study emphasize the importance of considering the circadian rhythmicity of cortisol, in relation to age, gender and (time of) stressor application, when studying the cortisol response of animals to stressors.

Introduction

In modern pig husbandry, animals are exposed to many stress factors that may affect their health and welfare. Especially, problems arise from environmental conditions such as housing and management factors. In studies on these problems, changes in concentrations of circulating corticosteroids, released from the adrenals, are often used as major physiological indicators of stress.

When studying the corticosteroid responses of animals to stressors, circadian (about 24 hour) fluctuations in basal corticosteroid concentrations have to be considered. In pigs, basal concentrations of cortisol in blood are generally higher in the morning than in the afternoon and evening (Barnett et al., 1981b; Bate and Hacker, 1985; Becker et al., 1985a; Evans et al., 1988; Griffith and Minton, 1991; Janssens et al., 1995; Klemcke et al., 1989). In addition to a span of high cortisol concentrations in the morning, in some studies, a peak is found in the afternoon

(Evans et al., 1988; Griffith and Minton, 1991). Moreover, differences in adrenocortical responses to stimuli may depend upon when, during the circadian cycle, a stimulus is applied (Ader and Friedman, 1968; Ottenweller et al., 1978). Exposure to stressors, on the other hand, may affect or disrupt the rhythm (chronobiological disturbance), as is reported for pigs (Barnett et al., 1981a; Bate and Hacker, 1985; Becker, 1990; Becker et al., 1985b; Janssens et al., 1995a) and rodents (Kawakami et al., 1972; Paris and Ramaley, 1974). As a result, the internal synchronization may be influenced, leading to chronic difficulties for the health and well-being of an organism (Turek, 1994).

The present study was designed to get more insight into the temporal dynamics of the circadian rhythm of cortisol with respect to the acute cortisol response to a stressor and, also, to study how the stressor may affect rhythmicity. The main objectives were therefore: (1) to document characteristics of basal cortisol concentrations in growing pigs in relation to age and gender; (2) to examine in growing pigs whether an acute stressor was able to affect or to disrupt the circadian rhythm of cortisol on the next day; and (3) to study the acute cortisol response to a stressor. For the latter two objectives, it was studied whether the outcome depended on age and on time of the day (at a phase of low or high basal cortisol concentrations) of stressor application.

For cortisol measurements, saliva was collected, which has proven to be an effective technique, causing minimum pain and distress with no need for restraint (Ekkel et al., 1996b; Parrott et al., 1989). The assessment of salivary cortisol has been shown to be a reliable and convenient endpoint of stress (Cook et al., 1996; Kirschbaum and Hellhammer, 1994). Salivary cortisol is mainly unbound and reflects the plasma free fraction (Fell et al., 1985; Kirschbaum and Hellhammer, 1994; Meulenberg, 1995; Riad-Fahmy et al., 1982; Vining et al., 1983). According to the free hormone concept, only the free hormone fraction is biologically active (Mendel, 1989). In pigs, (overall) cortisol concentrations in saliva are reported to be between 5 and 10% of those in plasma (Cook et al., 1996; Parrott et al., 1989). Assessments of salivary cortisol are relatively easy using sensitive immunoassay techniques.

Salivary cortisol rhythm characteristics were evaluated by the cosinor method, a tool in chronobiology (Bingham et al., 1982; Halberg, 1969; Nelson et al., 1979).

Materials and methods

Experimental housing and animals

The experiment was conducted at the experimental farm 'Bantham' from September to December. Five groups of fattening pigs, each consisting of 3 barrows and 3 gilts (crossbred; Great Yorkshire x (Great Yorkshire x Dutch Landrace)), were randomly selected from 17 litters at the age of 10 weeks and had previously been weaned at 4 weeks of age. Male piglets were castrated 2-4 days after birth. The groups were housed in pens (1.8 x 3.2 m) with fully slatted concrete floors until 25 weeks of age. The average (±SEM) body weight of the animals at 10 and 25 weeks of age was 25.7±2.4 and 99.7±9.3 kg, respectively. The pens were in a temperature- and humidity-controlled room with temperatures decreasing from 22.0±0.5 ^{0}C at 12 weeks to 18.4±0.6 ^{0}C at 24 weeks of age. Relative humidity varied between 60 and 80%. Main artificial lights were on from 6.00 until 20.00 h. Dim artificial lights were on continuously for the purpose of saliva collection and behavioural observations in the dark period. Total average lux of light at 35 cm above the floor was 52 in the daytime and 7 at night. Dim illumination of 7 lux was found not to interfere with the rhythm of cortisol in boars (Minton et al., 1989). Animals were fed commercial pelleted dry diets twice a day at 6.15 h in the morning and 15.15 h in the afternoon. They were allowed to eat for half an hour. Water was available ad libitum through a nipple drinker.

Experimental design

One week before each saliva collection for cortisol measurements, animals were accustomed to people and the sampling procedure, also during their sleep periods. When accustomed, we observed that, during sleep periods, pigs usually kept lying down and showed a minimum of extra physical activity. Each pig could individually be recognized by an ear tattoo and a number painted on the back. At the ages of 12, 16, 20 and 24 weeks, pigs from one group (control group) were repeatedly sampled every hour, to assess cortisol rhythmicity. At every age, sampling took place throughout two nonconsecutive 24-hour spans, on Monday and the following Friday. At the age of 12 weeks, animals in two other groups were separated from their pen mates (all animals from 1 group at the same time) and individually housed (isolated) in a smaller pen (1.8 x 1.6 m), either for a 4-hour span in the morning (8.00-12.00 h), when cortisol concentrations were anticipated to be high, or for a 4-hour span in the evening (18.00-22.00 h), when concentrations were expected to be low. During the isolation, animals were not able to have visual and tactile contact with other pigs. This type of isolation has been shown to be stressful by elevations of

free cortisol concentrations (Barnett et al., 1981a). At various times before and during application of the isolation stressor (-15, +20, +40, +60, +80, +100, +120, +180, +240 min) and after regrouping (+20, +40, +60, +90, +120 min), saliva was sampled to study the cortisol response. The stressor was applied on Thursday. Prior to and after the isolation stress, respectively on Monday and Friday, pigs from both groups were sampled for saliva every 2 hours for 24 hours to assess circadian rhythmicity. At the age of 20 weeks, this procedure was repeated for 2 other groups.

Saliva collection and cortisol analysis

Saliva was collected by simultaneous insertion of 2 veterinary cotton buds (Paul Hartmann, Nijmegen, The Netherlands) in the back of the mouth. The animals were allowed to chew for 1-2 min until the buds were thoroughly moistened. The buds were placed in special centrifuge tubes with inner cases (Sarstedt B.V., Etten-Leur, The Netherlands) and kept on ice until centrifuged for 5 min at 400 *g* to remove the saliva. Usually 1-2 ml of saliva was retrieved, which was then stored at -20^{0}C prior to cortisol analysis.

Concentrations of cortisol were measured in saliva samples, using a modified solid-phase radioimmunoassay for cortisol in plasma, serum and urine (Coat-A-Count Cortisol® TKCO, Diagnostic Products Corporation, Apeldoorn, The Netherlands). Samples were centrifugated for 5 min (2500 rpm, room temperature) to precipitate mucins. The procedure of the manufacturer was used with the following modifications: a buffer (0.05 M $Na_2HPO_4.2H_2O$, pH 7.3; 0.1% human albumin) was used to dilute the supplied human serum-based calibrators to final cortisol concentrations of 0.00 (maximum binding or B_0), 0.10, 0.25, 0.50, 1.25, 2.50, 5.00, 10.00 and 25.00 ng/ml. Saliva samples were assayed in duplicate and calibrators quadruplicate in amounts of 400 µl per assay tube (sample amounts lower than 400 µl were diluted quantitively with buffer). After overnight incubation on a shaking platform at room temperature, supernatants were aspirated from each tube. The remaining radioactivity in the tubes was measured for 1 min using a model 1470 Wizard® gamma counter (Wallac Oy, Turku, Finland). From the results of repeated determinations of 3 control (pooled saliva) samples, that were read at roughly 20, 50 and 80% of the maximum binding (B_0), intra-assay and inter-assay coeffients of variation were calculated. Determination by a 6-fold measurement of these samples in 4 assays with mean cortisol concentrations of 19.82, 5.85 and 0.75 ng/ml, resulted in mean (±SEM) intra-assay coefficients of variation of 3.6 (2.09), 3.8 (2.02) and 9.1 (2.8)%, respectively. The inter-assay coefficients of variation for the same saliva samples in these 4 assays were estimated as 7.8, 9.8 and 7.4%, respectively. Reco-

very of several amounts of calibrator cortisol, added to saliva, was determined to test accuracy. Addition of 0.50, 1.25, 2.50, 5.00 and 10.00 ng cortisol per ml of saliva, with an endogenous cortisol concentration of 0.45 ng/ml, resulted in recoveries of 117, 104, 104, 101 and 103%, respectively. Parallelity of the calibration curve with a saliva dilution curve (dilutions 1:2, 1:4, and 1:8 in buffer) was tested in quadruplicate for a sample of 5.27 ng cortisol per ml. In percentage of the expected concentrations, the mean measured value was 106%. This indicates that the procedure maintains good parallelity under dilution. The minimal detectable dose or sensitivity (concentration at 95% of the maximum binding) of the assay was approximately 0.13 ng cortisol per ml. Crossreactivities of the assay for other components that might be present in saliva are low. According to the manufacturer, corticosterone, cortisone, 11-deoxycorticosterone and 11-deoxycortisol, exhibit crossreactivities of 0.94, 0.98, 0.26, and 11.4%, respectively.

Statistical analysis

Analysis of circadian rhythmicity of salivary cortisol. Rhythm characteristics for cortisol were evaluated by the cosinor method (Bingham et al., 1982; Halberg, 1969; Nelson et al., 1979). Parameters such as the MESOR, amplitude, and acrophase were extracted. Moreover, residual standard deviations (σ; RSD) were estimated, representing variation around the individual curves. Cosine curves with periods of 24 hours were fitted per animal per day: $y_t = M + A \cos((2\pi/24)t + \phi) + \varepsilon_t$. Here, y_t is an observation at time t, M is the MESOR (**M**idline-**E**stimating **S**tatistic **O**f **R**hythm; the average level around which the oscillation occurs), A is the amplitude (measure of the extent of rhythmic change; maximum and minimum concentrations are M + A and M - A respectively) and ϕ is the acrophase (measure of the time at which the fitted cosine reaches its maximum value) expressed in radians ($-2\pi < \phi \leq 0$). The error term ε_t, within an animal, is assumed to be independently normally distributed with mean 0 and unknown constant variance σ^2. After re-parameterization, the model can be fitted by linear regression (Bingham et al., 1982; Nelson et al., 1979; Tong, 1976). To test for rhythmicity, the traditional F-test which compares the (re-parameterized) cosine model with the nonrhythmic model $y_t = M + \varepsilon_t$ was employed.

Estimated M, A, and ϕ, determined for each animal on each test day, were analyzed separately with a mixed analysis of variance model (48), e.g. $M_{ijk} = M + Group_i + Gender_l + Age_m + Weekday_p + Stressor\ application_q + animal_{ij} + day_{ijk} + e_{ijk}$. $Group_i$, ..., $Stressor\ application_q$ refer to systematic effects of the factors group, gender, age, day of the week (Monday or Friday), stressor application (not applied, applied on Thursday morning or Thursday evening). $Animal_{ij}$ is a random effect for

the j-th animal in group i and day_{ijk} is a random effect for the k-th day within the j-th animal in group i. Residuals e_{ijk} represent estimation error. The variance of e_{ijk} was fixed at the estimated variance of M_{ijk} from the corresponding fitted cosine curve. Components of variance and systematic effects were estimated by restricted maximum likelihood (REML) (Engel, 1990), employing the statistical programming language Genstat 5® (Genstat 5 Committee, 1993). Tests for interactions and main effects were based on the Wald-test (Buist and Engel, 1994; Rao, 1973).

Estimated residual variances σ^2 were analyzed with a generalized linear mixed model (Engel and Keen, 1994), employing a Genstat 5® procedure (Keen, 1994). Effects were introduced on the logarithmic scale. It was assumed that, for a given animal and 24 hour period, the variance $Var(\sigma^2) = 2\lambda\sigma^4/\nu$, where ν is the number of degrees of freedom (22 and 10 for observations per hour and per 2 hours, respectively) and dispersion factor λ was estimated from the data.

To determine to what extent animals may be characterized individually by their estimates M, A, and ϕ, the amount of variance between testdays 'explained' by the animals was calculated: $\{\sigma^2_{M\ animal} / (\sigma^2_{M\ animal} + \sigma^2_{M\ day})\} * 100\%$, where $\sigma^2_{M\ animal}$ and $\sigma^2_{M\ day}$ are the estimated components of variance of animals and days, respectively. Effects were considered significant if $p<0.05$. Data are presented as mean±SE, unless otherwise stated.

Analysis of acute cortisol responses. For each animal, 4 summary statistics were calculated and analyzed separately with an analysis of variance model with main effects and interaction for the factors gender, age and time of stressor application. During the isolation span, salivary cortisol concentrations were summarized as: (1) average over time-points 20 and 40 min after the start of the isolation minus the level at t = -15 min (UP1); and (2) regression gradient for time-points 20, 40, 60, 80, 100 and 120 min after the start of the isolation (DOWN1). Similar calculations were performed following the start of regrouping: (3) average over time-points 20 and 40 min after regrouping minus the level just before regrouping (UP2) and; (4) regression gradient for time-points 20, 40, 60, 90 and 120 min after regrouping (DOWN2). Because the summary statistics all involve contrasts between time-points within animals, it was assumed that possible group effects (which are confounded with stressor application and age effects) were largely eliminated. Effects were considered significant if $p<0.05$. Data are presented as mean±SE, unless otherwise stated.

Results

Circadian rhythmicity of salivary cortisol

Estimated individual rhythms. In Table 1, the estimates for the MESOR, amplitude, acrophase, and residual standard deviation (RSD), per animal, per 24 hour period, are summarized. The variation between the estimated values is the net result of variation between animals, between rhythms within animals (random test day variation), and estimation error. Standard errors of estimates, reflecting estimation error, are also listed in Table 1. The variation between these standard errors reflects the fact that for some animals the rhythm is apparent just by looking at a plot of the data, while for other animals the pattern is less obvious.

Table 1. Summary of parameter values, corresponding standard errors (SE), and residual standard deviations (RSD) of the circadian rhythm of salivary cortisol in individual pigs.

		Minimum	Mean	Maximum
MESOR	(ng/ml)	0.29	0.87	2.19
Amplitude	(ng/ml)	0.06	0.33	1.33
Acrophase	(rad)	-5.63	-3.01	-0.67
SE of MESOR		0.03	0.13	0.52
SE of amplitude		0.04	0.18	0.75
SE of acrophase		0.19	0.71	4.10
RSD		0.15	0.49	1.85

The cosinor method was used for analysis of rhythm parameters: MESOR (**M**idline-**E**stimating **S**tatistic **O**f **R**hythm), average level around which the oscillation occurs; amplitude, measure of the extent of rhythmic change; acrophase, measure of the time expressed in negative radians (rad; $-2\pi < \text{acrophase} \leq 0$) at which the fitted cosine reaches the maximum value. Residual standard deviations (RSD) represent variation around the fitted curves. Five groups of each 3 barrows and 3 gilts were involved in the experiment. In a control group, saliva samples were taken at 1-hour intervals at 12, 16, 20 and 24 weeks of age. Within one week, rhythmicity was assessed during two 24-hour periods (Monday and Friday). In 2 groups at 12 weeks and 2 other groups at 20 weeks of age, a stressor was applied (4 hours of isolation) on Thursday morning or evening. Again, rhythmicity was assessed on Monday and Friday but by sampling at 2-hour intervals.

At the 5% significance level, 40% of the F-tests for rhythmicity within animals were significant. At the 10% level, 56% of the F-tests were significant (comparable to the percentages given by Ekkel et al. (1996b). F-tests may be pooled over animals and in that case a very significant overall result was obtained. When extra terms for 6- and 12-hour periods were added to the linear regression model, tests for these extra periods were only incidentally significant. This offered no conclusive evidence for a more complex model consisting of a sum of cosines, each with a different period, or for a cosinor model with a smaller period. It was concluded that there was sufficient evidence for a biological rhythm to proceed with the mixed model analysis of variance.

Model selection for analysis of rhythm parameters. The final mixed model considered for the MESOR included all main effects and the significant statistical interaction between the factors age and stressor application. Main effects for the factors group, gender and weekday were all significant. For the amplitude, a significant interaction was found between the factors gender and stressor application, only when for the latter factor results for morning and evening were pooled. From the main effects, only those for the factors age and group were significant. For the acrophase, the interaction between the factors age and weekday was significant. From the main effects, the lowest *p*-value was for the factor stressor application (not significant; p=0.10). For the residual standard deviation, no significant interactions were found and a final model with main effects only was fitted. The main effects for the factors age and stressor application were both significant.

For the individual characterization of animals, the percentages 'explained by the animals' were too low. For the MESOR 66±13% and for the acrophase 77±13% of testday variation was explained by the animals. For the amplitude, estimated variance components for variation between animals and between testdays within animals were negligible compared with residual variation.

Effects of age. In Table 2, the cosinor parameter values at the different ages are shown. The MESOR of salivary cortisol decreased with increasing age. The differences in the MESOR were significant when compared pairwise, except for ages 20 and 24 weeks. For the amplitude, ages 12, 16 and 24 weeks did not differ significantly, whereas age 20 weeks was significantly below ages 12 and 16 weeks. Averaged over Monday and Friday, the circadian acrophase of salivary cortisol at age 12 weeks was significantly later than at ages 16, 20 and 24 weeks. The latter 3 ages did not differ significantly when compared pairwise. Estimated residual standard deviations at ages 12 and 16 weeks did not differ significantly nor did ages 20 and 24 weeks. The pooled residual standard deviation (0.53) for ages 12 and 16

was a factor 1.4 higher than that for ages 20 and 24 weeks (0.39). In Figure 1, fitted cosine curves for ages 12, 16, 20 and 24 weeks are shown, together with mean (±SEM) salivary cortisol concentrations, under basal conditions on Monday and averaged over genders.

Gender differences. Estimated values of circadian rhythm parameters of salivary cortisol for barrows and gilts are shown in Table 3. The overall mean MESOR for salivary cortisol in barrows is significantly higher than in gilts. For the amplitude, acrophase and residual standard deviation, effects of gender were not significant.

Table 2. Effect of age on parameter values (mean±SE) and on residual standard deviation (RSD±SE) of the circadian rhythm of salivary cortisol in growing pigs.

		Age (weeks)			
		12	16	20	24
MESOR[1]	ng/ml	1.19 ± 0.05^{A}	1.03 ± 0.06^{B}	0.74 ± 0.05^{C}	0.71 ± 0.06^{CD}
Amplitude	ng/ml	0.39 ± 0.03^{A}	0.39 ± 0.04^{A}	0.29 ± 0.03^{BC}	0.34 ± 0.03^{AC}
Acro-phase[2]	rad	-3.22 ± 0.17^{A}	-2.60 ± 0.23^{B}	-2.79 ± 0.18^{B}	-2.48 ± 0.21^{B}
	hours	12.18±0.39	9.56±0.53	10.39±0.41	9.28±0.48
RSD		0.53 ± 0.05^{A}	0.52 ± 0.06^{A}	0.36 ± 0.03^{B}	0.41 ± 0.05^{B}

For an explanation of parameters, see footnote of Table 1. The acrophase is also expressed in clock hours. ^{A,B,C,D}Means with different superscripts within the same row differ ($p<0.05$). [1]Interaction between the factors age and stressor application ($p<0.05$). Means under basal conditions are presented. [2]Interaction between the factors age and weekday ($p<0.05$). Estimated means are averaged over Monday and Friday.

Effects of stressor application. Estimated values of circadian rhythm parameters of salivary cortisol on the day after isolation are shown in Table 4. At 12 and 20 weeks of age, after isolation in the evening, the MESOR for salivary cortisol was higher than after isolation in the morning ($p>0.05$). However, when compared to the MESOR under basal conditions, at the age of 12 weeks, only isolation in the morning caused a significant change: a decrease in the MESOR. In contrast, at 20 weeks of age a significant effect of the stressor on the MESOR was only reached after application in the evening, revealed by a rise. After stressor application (pooled results for morning and evening; see Model Selection), the amplitude significantly increased in barrows but remained unchanged in gilts, reaching an almost significant

difference (p=0.06) between the genders. For the acrophase, although pairwise comparisons showed no significant differences, the difference between the mean under basal conditions and that after stress in the evening was nearly significant (p=0.06). Estimated means for residual standard deviations for basal conditions and after stressor application in the morning did not differ significantly, but both were significantly lower than after stressor application in the evening. The pooled residual standard deviation for basal conditions and stressor application in the morning, is 1.8 times lower when compared to the residual standard deviation after the evening stressor: 0.37 and 0.65, respectively.

Effects of other factors. Regarding weekday, the MESOR was found to be significantly higher on Monday. Averages for Monday and Friday were 0.99±0.04 and 0.87±0.04 ng cortisol per ml, respectively. Differences between weekdays were neither significant for the amplitude, nor for the acrophase, when averaged over the ages. Groups were found to be significantly different for the MESOR and amplitude, but not for the acrophase.

Table 3. Effect of gender on parameter values (mean±SE) and on residual standard deviation (RSD±SE) of the circadian rhythm of salivary cortisol in growing pigs.

		Gender	
		Barrows	Gilts
MESOR	(ng/ml)	1.01±0.05^{A}	0.86±0.04^{B}
Amplitude[1]	(ng/ml)	0.32±0.03^{A}	0.34±0.03^{A}
Acrophase	(rad)	-2.88±0.19^{A}	-2.66±0.19^{A}
	(hours)	11.00±0.44	10.10±0.44
RSD		0.47±0.04^{A}	0.42±0.04^{A}

For an explanation of parameters, see footnote of Table 1. The acrophase is also expressed in clock hours. ^{A,B}Means with different superscripts within the same row differ (p<0.05). [1]Interaction between the factors gender and stressor application (p<0.05; Morning and evening results are pooled for the factor stressor application). Means under basal conditions are presented.

Acute cortisol response

No significant interaction effects were found. For the main effects, effects of the factors age and gender were not significant. Main effects for the factor moment of stressor application, when averaged over ages 12 and 20 weeks, were significant for UP1 and UP2. For stress applied in the morning, UP1 and UP2 were 1.38±0.46

and 0.31±0.14 ng/ml higher, respectively, than for stress applied in the evening. No significant effects for DOWN1 and DOWN2 were found. As an additional check, average basal cortisol levels during the morning and evening stressor periods were compared. The levels were nearly significantly (p=0.08) or significantly higher during the morning periods when compared to those in the evening, at 12 and 20 weeks of age, respectively.

Table 4. Effect of isolation stress on parameter values (mean±SE) and on residual standard deviation (RSD±SE) of the circadian rhythm of salivary cortisol in growing pigs.

		Isolation on previous day			
		No	Morning	Evening	Pooled
MESOR[1]	ng/				
12 weeks	ml	1.19±0.05^{A}	0.96±0.10^{B}	1.15±0.12^{AB}	
20 weeks		0.74±0.05^{A}	0.77±0.09^{AB}	0.91±0.09^{BC}	
Amplitude[2]	ng/				
Barrows	ml	0.32±0.03^{A}			0.44±0.05^{B}
Gilts		0.34±0.03^{A}			0.32±0.04^{A}
Acrophase	rad	-3.07±0.18^{A}	-2.77±0.25^{A}	-2.47±0.27^{A}	
	hours	11.44±0.41	10.35±0.57	9.26±1.02	
RSD		0.41±0.04^{A}	0.33±0.05^{A}	0.65±0.09^{B}	

For an explanation of parameters, see footnote of Table 1. The acrophase is also expressed in clock hours. ^{A,B,C}Means with different superscripts within the same row differ (p<0.05). [1]Interaction between the factors age and stressor application (p<0.05). [2]Interaction between the factors gender and stressor application (p<0.05; Morning and evening results are pooled for the factor stressor application).

Discussion

By using the cosinor analysis as performed in this study, we were able to describe the temporal dynamics of circadian rhythms of salivary cortisol by parameters such as the MESOR, amplitude and acrophase. Our data document changes in basal salivary cortisol concentrations in growing pigs, influenced by age and gender. Moreover, after isolation stress, changes in cortisol rhythm characteristics on the next day were revealed, depending on age and (time of) stressor application. The acute cortisol response was affected by time of stressor application.

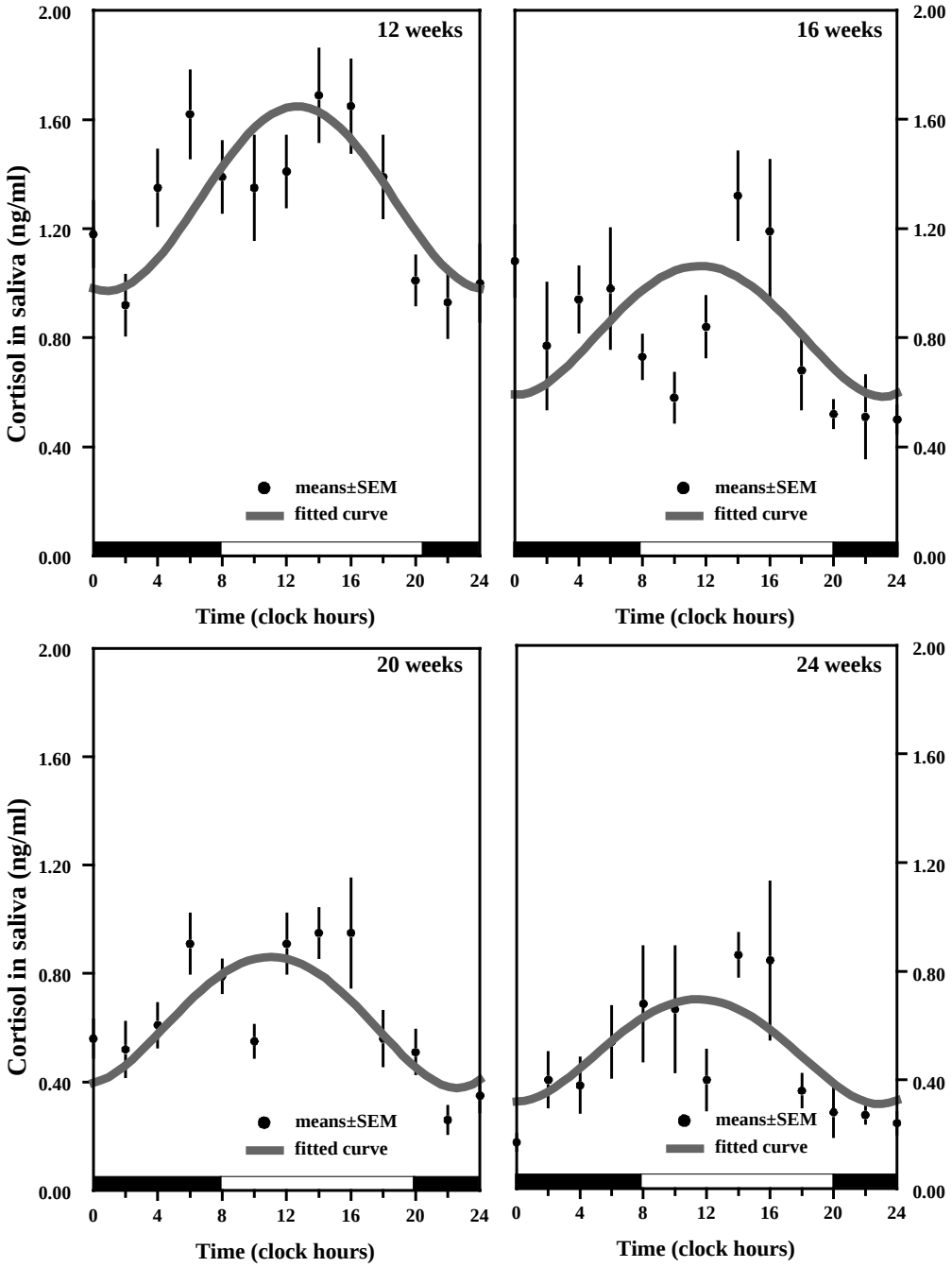

Figure 1. Mean(±SEM) salivary cortisol values under basal conditions on Monday (•), together with fitted cosine curves (shaded lines), in growing pigs from 12 to 24 weeks of age. For all ages, animals of the Control group (n = 6) were included. Also, at the age of 12 weeks animals from 2 stress groups (both n = 6), and at the age of 20 weeks animals of 2 other stress (isolation) groups (both n = 6), were included.

Compared with other studies reporting that, in pigs, concentrations are highest in the morning in both blood (Barnett et al., 1981b; Bate and Hacker, 1985; Becker et al., 1985a; Evans et al., 1988; Griffith and Minton, 1991; Janssens et al., 1995a; Klemcke et al., 1989) and saliva (Ekkel et al., 1996b, 1997), we found peak concentrations of salivary cortisol in the late morning and early afternoon. In the growing animals, an effect of age on circadian salivary cortisol profiles was observed. Between 12 and 24 weeks of age, basal salivary cortisol concentrations decreased, shown by a decrease of the MESOR and a slight decrease in the amplitude (Figure 1; Table 2). Our results are in agreement with those of Evans et al. (1988) and Kirkwood et al. (1987), who reported a decrease or a tendency for a decrease with age for (total) cortisol concentrations in plasma in growing pigs. Parallel human studies also show a decrease in the MESOR of cortisol in children aging from 6-12 months until 15 years (Haen et al., 1984; Onishi et al., 1983). A shift in plasma cortisol from rather unbound forms to that bound to corticosteroid-binding globulin (CBG), was found to occur in pigs from birth to 6 weeks of age (Kattesh et al., 1990). This process of increased binding of cortisol to CBG, when continued after the age of 6 weeks, may explain our findings. Our results are not supported by Ekkel et al. (1997) who found a higher MESOR and a greater amplitude for the circadian rhythm of salivary cortisol in 15-week old pigs when compared to 10-week old pigs. Moreover, it was shown in children that, after an initial increase, the circadian amplitude stabilized at an adult level at 1-6 years of age (Haen et al., 1984; Onishi et al., 1983). In our study, effects of age on the other parameters was reflected by a shift in acrophase to an earlier time and a decrease in estimated variation around the fitted curves.

In very young pigs, circadian cortisol rhythms and other circadian rhythms may be weak (Ingram et al., 1985) or absent (Evans et al., 1988), a phenomenon also shown in very young children (Haen et al., 1984; Onishi et al., 1983). However, a circadian rhythm of cortisol in saliva was demonstrated in 8-weeks old pigs by Ekkel et al. (1996b). Evans et al. (1988) reported a gradual development of a distinct circadian rhythm of (total) cortisol in plasma in growing pigs, reaching an adult profile near puberty. Our results indicate that the age of 20 weeks may be important for reaching adult circadian rhythm profiles of salivary cortisol. At this age, the MESOR, amplitude, and variation around the fitted curves were shown to stabilize or to be stabilized. The stabilization of the acrophase already occurred at 16 weeks of age.

Gender-related differences in cortisol rhythm parameters were only found for the MESOR, with a higher value for barrows than for gilts. Although differences in plasma (total) cortisol between barrows and gilts are not reported (Marple et al.

1974; Nyberg et al., 1988), Marple et al. (1974) found higher concentrations in boars than in gilts. Nyberg et al. (1988) found a tendency towards higher CBG concentrations in plasma of gilts than in barrows, which might result in lower unbound (and consequently lower salivary) cortisol concentrations in gilts. Alternatively, enhanced basal salivary cortisol concentrations in barrows may result from castration at 2-4 days after birth, possibly experienced as a major stress by the animals. It has been demonstrated in rats that early experiences in life can induce long-term changes in the regulation of the HPA-axis (Meaney et al., 1988).

After isolation stress, on the following day the amplitude of the circadian rhythm of salivary cortisol was elevated in barrows, but there was no change in gilts. This increase in barrows may also be attributed to castration, causing sensitization and facilitation of HPA responses. However, these results are in contrast with findings in rats, where (brief social) stress caused a decrease of the amplitudes of the circadian rhythms of heart rate and core temperature (Tornatzky and Miczek, 1993). After isolation in the evening, the variation of the rhythm around the fitted curves was higher when compared to results after morning stress and under basal conditions. The acrophase tended to shift when compared to that under basal conditions. These differences may be due to the time interval between application of the stressor and the determination of the rhythm the next day, which was much shorter for evening isolation. This may also apply for the MESOR, which tended to be higher after evening isolation than after morning isolation, both at 12 and 20 weeks of age. However, at 12 weeks of age, the MESOR following stress was suppressed after morning isolation but was at basal level after evening isolation. At 20 weeks of age, the MESOR the following day was at basal level after morning isolation and elevated after evening isolation. These differences may be explained by differences in feed-back action of cortisol depending on age (Goldman et al., 1973), time of the day (Akana et al., 1986) and pattern (magnitude and duration) of the acute cortisol response (Engeland et al., 1977). For short term stressors, the return to resting cortisol concentrations (in blood) takes about 24 hours for cannulation (Becker et al., 1985a) and at least 48 hours after food and water deprivation or water deprivation alone (Becker, 1990). Long-term alterations in the circadian rhythm of cortisol (in blood) have been reported under chronic stress conditions such as individual penning (Barnett et al., 1981a), tethering (Becker et al., 1985b; Janssens et al., 1995a), and exposure to different ambient temperatures (Bate and Hacker, 1985).

The acute cortisol response was affected by time of the day of stressor application. The overall acute cortisol response during isolation and following regrouping was higher in the morning, when basal cortisol concentrations were already high,

than in the evening, when basal cortisol concentrations were low. This was shown by higher 'UP' values of salivary cortisol. Circadian variation in the responsiveness of the adrenals to a stressor has been the subject of many studies in different species, but the results are conflicting. No differences in cortisol responses (in plasma) were observed between morning and afternoon application of stressors (32^0C for 2 hours or 20-min restraint) in barrows (Kirkwood et al., 1987). However, comparison of more divergent time-periods could have given other results. In addition to that, in this type of experiments, it may be of interest to use a design relying on more than two time-points at which a stressor is applied (Günther et al., 1980). From studies in rats (Ader and Friedman, 1968; Engeland et al., 1977), it is concluded that differences in acute stress responses during the day are depending on the intensity and/or duration of the stressor. Interestingly, circadian variation in adrenal sensitivity to ACTH has been suggested in pigs (Klemcke et al., 1989) and rats (Dallman et al., 1978; Engeland et al., 1977; Nicholson et al., 1985). In pigs, this was shown by a greater cortisol/ACTH ratio in the morning than in the evening (Klemcke et al., 1989). This might explain our findings of a higher acute cortisol response to the isolation in the morning compared to that in the evening.

Differences between weekdays were found for the MESOR, which was higher on Monday than on Friday. One explanation for this is the occurrence of a circaseptan or weekly cortisol rhythm, although the existence of circaseptan rhythms is questioned by Turek (1994). Differences may also be imposed by, for example, differences in management between weekdays, although minimized as much as possible. To some extent, animals might also be more adapted to saliva collection on Fridays compared with Mondays.

In conclusion, the results of this study demonstrate the development of a circadian rhythm of cortisol in the saliva of growing pigs, reaching an adult and rather stable profile around 20 weeks of age. Furthermore, the average concentrations of cortisol decreased with age and were higher in barrows than in gilts. The circadian rhythmicity of salivary cortisol was changed after applying isolation stress, depending on age, gender and time of stressor application. Also, the acute salivary cortisol response was affected by time of day of stressor application. Thus, when using (salivary) cortisol as a physiological indicator of stress, this study emphasizes the importance of considering the effects of age, gender and (time of) stressor application.

Chapter 3

Housing familiar male Wildtype rats together reduces the long-term adverse behavioural and physiological effects of social defeat

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M.A.W. Ruis[1,2], J.H.A. te Brake[1], B. Buwalda[2], S.F. de Boer[2], P. Meerlo[2], S.M. Korte[1], H.J. Blokhuis[1], J.M. Koolhaas[2]

[1] Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB Lelystad, The Netherlands
[2] Department of Animal Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

Abstract

Social stress in rats is known to induce long-lasting, adverse changes in behaviour and physiology, which seem to resemble certain human psycho-pathologies, such as depression and anxiety. The present experiment was designed to assess the influence of individual or group housing on the vulnerability of male Wildtype rats to long-term effects of inescapable social defeat. Group-housed rats were individually exposed to an aggressive, unfamiliar male conspecific, resulting in a social defeat. Defeated rats were then either individually housed or returned to their group. The changes in their behaviour and physiology were then studied for 3 weeks. Results showed that individually housed rats developed long-lasting, adverse behavioural and physiological changes after social defeat. Their body growth was significantly retarded ($p<0.05$) between 7 and 14 days after defeat. When individually and group-housed rats were exposed to a mild stressor (sudden silence), 2 days after defeat, both groups became highly immobile. However, when exposure was repeated at day 21, individually housed rats were still highly immobile compared to group-housed rats which regained their normal mobility after only 7 days. In an open field test, also regularly repeated, individually housed rats took significantly longer to leave their home base and were also significantly less mobile than group-housed rats over the entire 3-week test period as well as at specific timepoints. When the rats were placed in an elevated plus-maze 14 days after defeat, those that were individually housed were significantly more anxious than those that were group-housed. When tested at 21 days after defeat in a combined dexamethasone (DEX)/corticotrophin-releasing factor (CRF) test, results showed that the hypothalamic-pituitary-adrenocortical (HPA) activity in individually housed rats was higher. This was evidenced in the latter animals by the fact that DEX was significantly less able to suppress the secretion of ACTH and corticosterone, and by a significantly higher release of ACTH after administration of CRF. Although the weights of the spleen and testes of the two groups did not differ, the adrenals of individually housed rats were larger and the thymus and seminal vesicles were smaller. We conclude that when rats are isolated after defeat, they show long-lasting, adverse behavioural and physiological changes that resemble symptoms of stress-related disorders. In contrast, when familiar rats are housed together these effects of a social defeat are greatly reduced. These findings show that housing conditions importantly influence the probability of long-term adverse behavioural and physiological effects of social defeat in male wildtype rats.

Introduction

Major life events have been shown to be involved in the development of stress-related disorders in humans, including affective disorders such as depression and anxiety (Biondi and Picardi, 1996; Brown, 1993). The physiological mechanisms underlying psychopathologies have not only been directly investigated in human patients, but also indirectly in animal models. Although few animal studies have evaluated the effects of single life events, recent studies in rats demonstrate that long-lasting, adverse effects arise after a single social defeat (Koolhaas et al., 1997b; Miczek et al., 1990).

Social defeat has been shown to cause several behavioural and physiological changes in rats. Although the time courses of these changes differ greatly (Koolhaas et al., 1997b), there are several long-lasting effects of social defeat. These are, for example, retardation of body growth (Meerlo et al. 1996c, 1997), reduced mobility in response to a mild stressor (Koolhaas et al., 1990), decreased open field activity (Meerlo et al., 1996b), high anxiety in a plus-maze test (De Boer et al., unpublished data), and increased hypothalamic-pituitary-adrenocortical (HPA) activity (Buwalda et al., 1999). Koolhaas et al. (1990, 1997b) have suggested that the social defeat model in rats seems to be a promising model for human depression, but the interpretation of long-lasting changes following social defeat in rats in terms of pathologies similar to those in humans is still an important fundamental issue. However, the fact that certain behavioural and physiological changes can be counteracted by depriving rats of sleep (Koolhaas et al., 1990; Meerlo et al., 1996b) and by the use of antidepressants (Fuchs et al., 1996; Koolhaas et al., 1990; Sampson et al., 1991) shows that stress disorders exist in animals that may resemble those in humans.

However, the social defeat model in rats was developed using rats that were individually housed, and until now it has not been known whether rats that are housed with familiar congeners would also develop long-lasting, adverse behavioural and physiological changes. Wild rats live in groups and we speculate that the presence of familiar group members is beneficial for health and well-being by moderating or buffering rats against the adverse effects of stress (social support). A number of health studies in humans have also shown that social support reduces the frequency of depression (Biondi and Picardi, 1996; Coyne and Downey, 1991; Elmore, 1984, Miller and Surtees, 1995, Paykel, 1994) and the incidence of disease-related mortality and morbidity (Cohen, 1988). However, little has been published on this subject related to animals and results are contradictory. Although Van Dijken et al. (1992) found that housing rats in groups did not

moderate the pathological effects of foot-shocks, Boccia et al. (1997) suggested that social support in nonhuman primates did lessen adverse effects of stress.

The purpose of our study was to assess the influence of individual or group-housing on the vulnerability of rats to the long-term adverse effects of an inescapable social defeat stress. Group-housed rats were exposed to social defeat by an aggressive noncongener, after which they were either individually housed (social isolation) or returned to their familiar group (group-housed). For 3 weeks after defeats, rats were weighed regularly and exposed to various behavioural and physiological tests. At the end of the 3-week period, part of the rats was killed and their adrenals, thymus, spleen, testes and seminal vesicles were removed for determinations of weights.

Materials and methods

Animals and housing

The experiment was performed with 24 male Wildtype rats, originating from 16 socially housed groups, each consisting of 3 littermates. Each group was housed in a cage of 40 x 30 x 20 cm. The rats were bred in the laboratory in Haren, were approximately 4 months old and weighed 399±6.6 g (mean±SEM) at the start of the experiment. Room temperature was maintained at about 21 ^{0}C. The light/dark cycle (12 hours/12 hours) was reversed (lights on from 20.00 until 08.00 h). Food and water were available ad libitum. After social defeat, the rats were either returned to their groups or were housed individually (cage size: 25 x 25 x 30 cm; clear Plexiglas). All experimental procedures took place in the dark (active) phase between 10.00 and 15.00 h. Cages were cleaned once a week and at least 2-3 days before experimental procedures.

Social defeats and housing conditions

Rats were removed from their social groups and transferred to a test room. In this room, previously selected aggressive male rats were housed in large cages (80 x 55 x 40 cm), along with one sterilized female. In a resident-intruder paradigm, an experimental rat (intruder) was introduced into the home cage of an unfamiliar aggressive rat (resident), from which the female was removed, for a standard period of 1 hour (Meerlo et al., 1996a,b,c). Experimental rats were attacked and defeated as indicated by fleeing, freezing and submissive behaviour. When a full submissive posture occurred, with the experimental rat lying motionless on its back, the animal was protected from further attacks by placing it in a small wire mesh cage (30 x 15 x 15 cm), within the home cage of the resident,

for the rest of the hour. This allowed auditory, olfactory and visual contact between intruder and resident. Defeat procedures took place under dim red light conditions. The defeated rats were returned either to their familiar groups or were housed individually. For each treatment, 12 rats were randomly chosen from 8 different social groups

Cannulations

A total of 1 week after the social defeats, half of the animals (n = 6 for each treatment) received a silicon heart catheter (0.95 mm OD, 0.5 mm ID), enabling blood samples to be collected in freely moving rats. The catheter was inserted through the right jugular vein, under halothane anasthesia, according to Steffens (1969). The external part of the cannula of the group-housed rats was protected by a small metal cap. Immediately after surgery, the rats were injected subcutaneously with 100 000 IE sodium-penicillin® G (Yamanouchi Pharma B.V., The Netherlands). The antibiotic treatment was repeated at 3 and 7 days after surgery.

Behaviour and body weight

Changes in the behaviour of non-cannulated rats (n = 6 for each treatment) were tested before social defeat and for 3 weeks thereafter. This was done 2 days before, and 2, 7, 14 and 21 days after defeat, with repeated open field and sudden silence tests. At day 14 after defeat an elevated plus-maze test was also performed. Body weights were measured on the days of behavioural testing.

Sudden silence test. Rats were placed in a large perspex cage (60 x 30 x 40 cm), within a soundproof wooden box with dim white lights (5 lux) and a glass front enabling observation. Each test consisted of two trials performed on 2 consecutive days. In the first trial, the rats were exposed to a constant 70 dB background noise for 5 min. During the last 3 min, the relative duration (% of time) of immobility was derived from direct recording with a keyboard. In the second trial (at the above mentioned time-points), the same procedure was followed, except that the 70 dB background noise was switched off after 2 min. Again, immobility was recorded during 3 min. The response to the sudden silence was considered to be the difference in immobile behaviour between trial one (with the noise) and trial two (without the noise). The test was described and validated by Koolhaas et al. (1990).

Open field test. Group-housed rats were temporarily separated from their groups and housed in individual cages from 1 day before the test until shortly after the test. After being transferred to a test room, the rat with its cage was put into the

centre of a round arena. This wooden arena was divided in two concentric zones, the inner zone with a diameter of 60 and the outer zone with a diameter of 120 cm, and was surrounded by a 30 cm high wall. At the start of the test, the cover of the cage was removed, leaving the rat on the bottom of the cage, representing the home base. The rat was allowed to explore the arena for 5 min. Behaviour was recorded with a camera and automatically analyzed with the software programme Ethovision® (Noldus Information Technology, Wageningen, The Netherlands). The following parameters were determined: (1) the latency period that elapsed before the rat left the home base to enter the outer zone; (2) locomotion, as expressed by the distance travelled in the outer zone; and (3) percentage of time spent in the outer zone (time in outer zone/(time in inner zone + time in outer zone) x 100). The test was performed under dim white light conditions (6 lux).

Elevated plus-maze test. The maze was a black wooden plus-shaped construction, elevated to a height of 50 cm. It consisted of two open arms (50 x 10 cm) opposite to each other, and two closed arms (50 x 10 x 40 cm; with an open roof), also opposite to each other (Korte et al., 1995; Pellow et al., 1985). A desk lamp was placed on one side of the maze so that the open arms were lighted, but closed arms remained dark. The light intensity on the open arms varied, ranging from 10 lux close to the center of the maze to 80 lux on the end of the open arms. In the closed arms it was less than one lux. Rats were placed individually in the center of the maze facing a closed arm. The number of entries into open and closed arms was scored with a keyboard for 5 min. In addition, the percentage time spent on open arms (time on open arms/(time on open arms + time on closed arms) x 100), the percentage entries into open arms (entries open/total entries x 100), and total entries were determined.

Organ weights

A total of 3 weeks after the social defeats, non-cannulated rats were killed and the adrenals, thymus, spleen, testes and seminal vesicles were removed and weighed. Organ weights were expressed relative to body weights (mg/100 g body weight).

Combined DEX/CRF challenge test and blood sampling

HPA activity was tested in a combined dexamethasone (DEX)/ corticotrophin-releasing factor (CRF) challenge test. First the capacity of DEX to suppress the secretion of corticosterone and ACTH was determined, then the responsiveness of these two hormones to CRF was determined (Hatzinger et al.,

1996; Heuser et al., 1994). The test was performed 21 days after the social defeats. Just before the test, group-housed cannulated rats were temporarily transferred to individual cages for the collection of blood samples. Individually housed cannulated rats were also put in new and clean individual cages. Immediately after transfer, rats were connected with a polyethylene blood sampling tube (40 cm length, 1.45 mm OD and 0.75 mm ID). DEX® (Sigma, St. Louis, MO) was injected SC to suppress HPA activity, in a concentration of 25 µg/kg, dissolved in a volume of 1 ml/kg of 50% polyethylene glycol in saline. At 89 min after DEX injection (1 min prior to CRF administration), a blood sample was collected to determine basal ACTH and corticosterone concentrations. Blood samples were drawn in amounts of 0.5 ml. Immediately thereafter, CRF® (oCRF, American Peptide, Sunnyvale, CA; 0.5 µg/kg per ml saline) was administered IV and blood samples were collected at 5, 15, 30, 60 min thereafter to measure hormone responses to CRF. Blood samples were immediately stored in chilled centrifuge tubes containing 0.3 % EDTA as anticoagulant and 30 µl aprotinin (10 000 KUI/ml) as protease inhibitor. Blood was centrifuged at 4^0C for 10 min at 2600 *g*. Amounts of 100 µl and 75 µl of the supernatants were stored at -20^0C, awaiting ACTH and corticosterone measurements, respectively. A two-site radioimmunoassay (Nichols Inst. Diagnostics, CA) with an intra-assay variance of 3.2% and an inter-assay variance of 7.8% was used to measure plasma ACTH. Reversed phase high performance liquid chromatography (HPLC) was used to measure plasma corticosterone, as described by Dawson et al. (1984). The detection limit of the assay was 0.8 µg corticosterone/100 ml, and the intra-assay and inter-assay variances were, respectively, 3 and 8%.

Statistical analysis

Data on body weights, levels of passivity in the repeated sudden silence test, and latencies and locomotions in the repeated open field test, were analyzed with a split-plot analysis of variance model for the repeated measurements (Snedecor and Cochran, 1967). After fitting the model to the data, residuals (predicted error terms within animals) were inspected for homogeneity of variance, which is an important model assumption. Data on body weights and on latencies and locomotions in the open field tests showed heterogeneity, mostly due to an increase of variance with an increasing mean. They were logarithmicly transformed and re-analyzed, i.e. treatment effects were expressed by multiplicative factors between treatment means, rather than by their differences. Fixed effects in the split-plot model were main effects and interactions for the factors housing (individually

or group) and time relative to social defeat (-2, 2, 7, 14 and 21 days). Random effects were animal specific effects and residual environmental effects, which account for variation between and within animals. Initially, a first order auto-regressive process was assumed for the residual terms within animals (Chatfield, 1989). Because differences between split-plot analyses with or without residual auto-correlation were negligible, results are only shown for split-plot analyses without residual auto-correlation. As a follow up, estimated means were compared with Fisher's Least Significant Difference method (LSD method; Snedecor and Cochran, 1967). One-way analysis of variance was performed to test the effect of housing on plus-maze behaviour, organ weights, and some characteristics of the hormone responses in the DEX/CRF tests. All calculations were performed with the statistical programming language Genstat 5® (1993), employing some of the restricted extended maximum likelihood (REML) facilities from the beta-test version of release 4.1 to fit an additional auto-regressive process. Effects were considered significant if $p<0.05$. Unless stated otherwise, data are presented as mean (±SEM).

Results

Body weight

At the start of the experiment, the mean body weight of the animals being individually housed was higher than that of animals being group-housed (418±15.9 vs. 380±12.7 g; NS). Therefore, body weights were expressed as percentage change relative to the weight shortly before the start of experimental procedures (Figure 1). Averaged over the entire 3-week period, the way that rats were housed significantly affected body weight gain, shown by a significant main effect for the factor housing ($p<0.05$). Body weight gain was lower in individually housed rats. When analyzed within separate timepoints, individually housed rats had gained significantly less weight at 7 and 14 days after defeats, but caught up with their group-housed counterparts between 14 and 21 days after defeats.

Behavioural observations

Immobility in the repeated sudden silence test. The type of housing significantly influenced durations of immobility when averaged over the 3-week period, shown by a significant main effect for the factor housing ($p<0.001$). Relative durations of immobility were 31.2±2.1 and 16.3±2.4% for individually and group-housed rats, respectively. Moreover, a significant interaction was found between the factors housing and time ($p<0.001$).

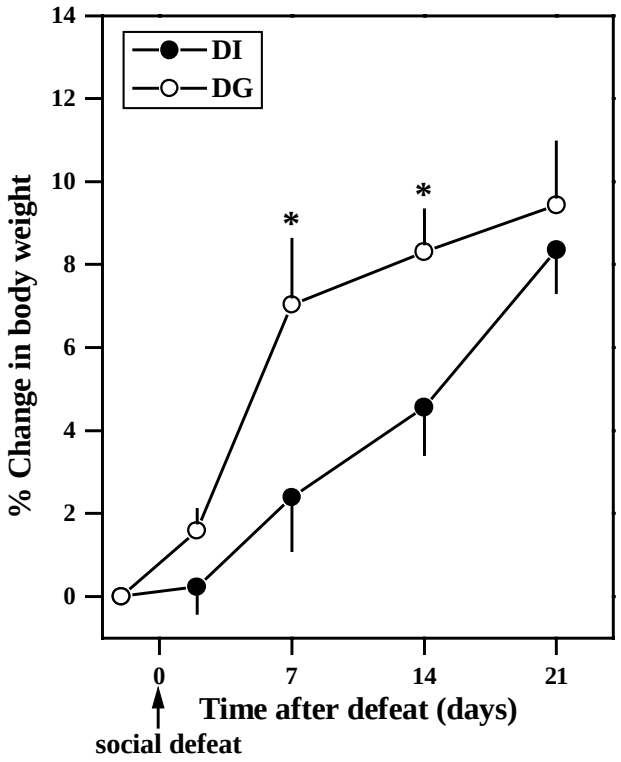

Figure 1. Time courses of changes in body weight. The change was expressed as percentage change (mean±SEM), relative to the weight shortly before the start of the experimental procedures: DI: defeat, individual housing (n = 6); and DG: defeat, group-housing (n = 6). Significant main effect for the factor housing (p<0.05). *Asterisks indicate significant differences (p<0.05) between the two groups.

Immobility increased significantly 2 days after the defeats for both individually housed rats and group-housed rats. However, at day 21 individually housed rats were still highly immobile (even a gradual increase) compared to group-housed rats which regained their normal mobility after only 7 days (Figure 2).

Latency period and locomotion in the repeated open field test. The average latency period (during the 3-week period after defeat) to leave the home base, lasted a factor 1.72±0.48 longer for individually housed rats when compared to their group-housed counterparts (significant main effect for the factor housing: p<0.05). By day 21, the difference between the two groups was significant (Figure 3A). A general decrease in latency times in the course of 3 weeks, was responsible for a main effect for the factor time (p<0.05).

The type of housing did not affect the amounts of time that rats spent in the outer zone of the open field. However, during the 3-week period after defeat, group-housed rats moved a factor 1.23±0.07 more in the outer zone than individually housed rats (significant main effect for housing: $p<0.01$). Within the different timepoints, differences between both groups were significant at 2 and 21 days after defeat (Figure 3B). In the course of weeks after defeat, locomotions decreased in both groups, shown by a significant main effect for the factor time ($p<0.001$).

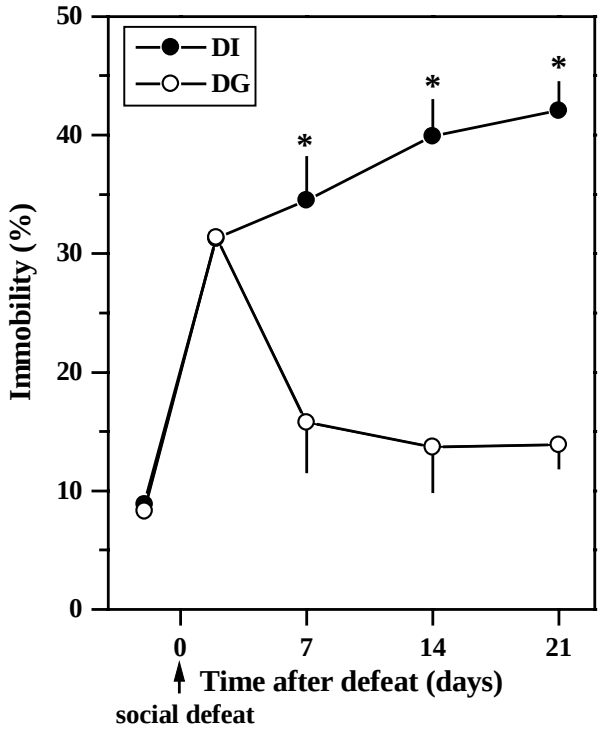

Figure 2. Time courses of changes in responses to a sudden silence. Immobility responses to a sudden silence were determined as the change (mean±SEM) from baseline values observed during exposure to the noise. DI: defeat, individual housing (n = 6); and DG: defeat, group-housing (n = 6). Significant interaction between the factors housing and time ($p<0.05$), and a significant main effect for the factor housing ($p<0.05$). *Asterisks indicate significant differences ($p<0.05$) between the two groups.

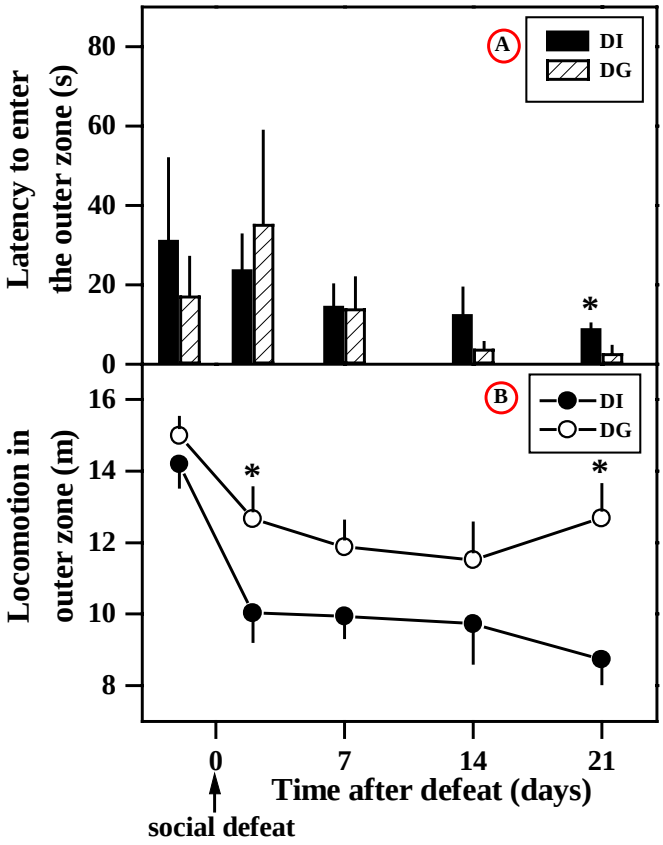

Figure 3. Time courses of changes in open field behaviour. DI: defeat, individual housing (n = 6); and DG: defeat, group-housing (n = 6). (A) Latency period that elapsed before the home base was left to enter the outer zone; (B) Total travelled distance in the outer zone. Data are expressed as mean±SEM. Significant main effects for the factors housing and time ($p<0.05$). *Asterisks indicate significant differences ($p<0.05$) between the two groups.

Times on and entries into the arms of the elevated plus-maze test. The type of housing significantly affected plus-maze behaviour when tested at 14 days after defeat (Figure 4). Individually housed rats spent less time on the open arms ($p<0.01$) and made fewer entries into the open arms ($p<0.01$). The total number of entries were about the same for individually and group-housed animals: 12±1 and 14±2, respectively.

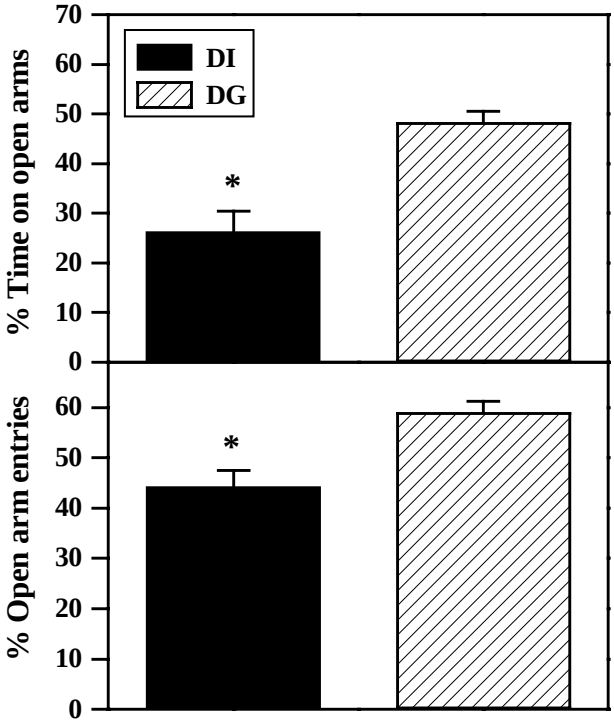

Figure 4. Mean (±SEM) percentage of time on open arms and percentage open arm entries on an elevated plus-maze at 14 days after social defeats and the start of different housing procedures. DI: defeat, individual housing (n = 6); and DG: defeat, group-housing (n = 6). *Significant difference (p<0.05) between the two groups.

HPA-axis activity

The way in which rats were housed, significantly affected HPA responses to DEX in the combined DEX/CRF test 21 days after social defeat (Figure 5). At 89 min after administration of DEX (1 min prior to the CRF challenge), ACTH (p<0.01) and corticosterone concentrations (p<0.01) were lower in the group-housed than in the individually housed rats. The way in which rats were housed also significantly affected the release (response: t = 5 minus t = -1 min) of ACTH when CRF was administered (p<0.05). A total of 5 min after administration, group-housed rats released less ACTH than individually housed rats. However, corticosterone responses did not significantly differ between the two groups. A total of 15 min after administration of CRF, no significant differences in the decline (t = 5 minus t = 15 min) of ACTH and corticosterone concentrations between the two groups were observed. Type of housing had a tendency (p=0.06) to affect the areas under the curves (AUC) for ACTH concentrations. Group-

housed rats had a lower AUC (660±144) than individually housed rats (1070±228). AUC for corticosterone concentrations did not differ.

Organ weights

As shown in Table 1, at 3 weeks after social defeat, the way in which rats were housed significantly affected the weights of the adrenals ($p<0.05$), thymus ($p<0.05$), and seminal vesicles ($p<0.01$). The adrenals of individually housed rats were larger, but their thymuses and seminal vesicles were smaller. Weights of testes and spleens did not differ between the two groups.

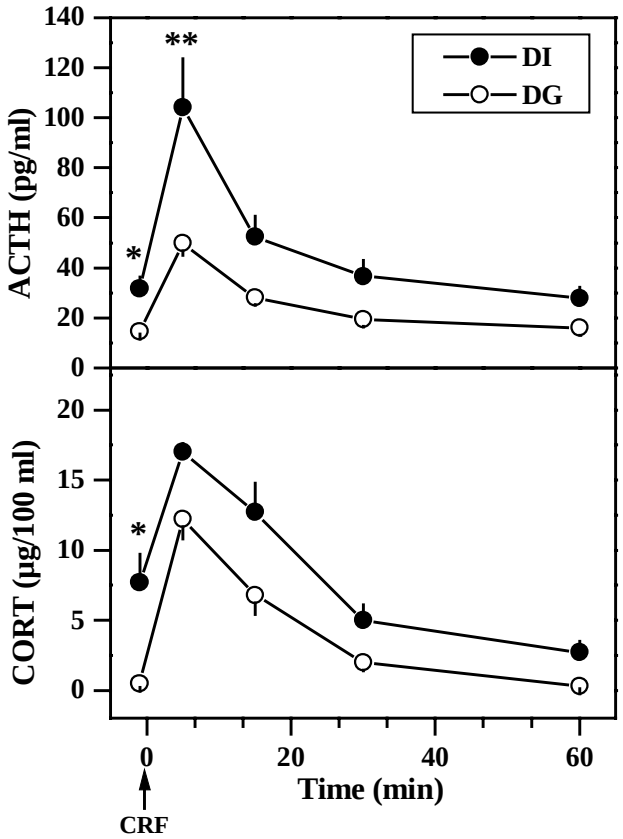

Figure 5. Mean (±SEM) plasma levels of ACTH and corticosterone (CORT) in a combined DEX/CRF test at 21 days after social defeats and the start of different housing procedures. DI: defeat, individual housing (n = 6); and DG: defeat, group-housing (n = 6). Animals were injected with DEX, 90 min prior to the CRF challenge. *Significant difference ($p<0.05$) in ACTH and corticosterone concentrations at -1 min, which was 89 min after DEX administration. **Significant difference ($p<0.05$) between the two groups in ACTH responses, 5 min after administration of CRF.

Table 1. Relative weights of various organs 21 days after social defeat and the start of different housing procedures (mean±SEM).

	Type of housing	
	Individually (n = 6)	Group (n = 6)
Adrenals (mg/100g)	14.3±0.5	12.8±0.3*
Thymus (mg/100g)	74.5±2.2	85.5±2.8*
Spleen (mg/100g)	252±9.8	222±18.5
Testes (mg/100g)	632±50.9	703±21.8
Seminal vesicles (mg/100g)	236±8.0	271±7.6*

*Asterisks indicate significant differences ($p<0.05$) between means.

Discussion

The results of this study show that the duration and severity of behavioural and physiological effects of a single social defeat depend on the way in which animals are subsequently housed. Long-term adverse effects of social defeat were greatly reduced in group-housed rats, when compared to those in defeated and individually housed rats.

Body growth of individually housed rats was significantly retarded in the first 2 weeks after defeat compared to rats that were reintroduced in their social groups (Figure 1). However, growth rate recovered and they caught up with the group-housed rats at 3 weeks after defeats. Meerlo et al. (1996c, 1997) also reported a low body weight gain in defeated and individually housed rats, recovering after about 5 days, but not catching up with isolated controls within 3 weeks. The retardation of growth may be at least partly due to the fact that food intake may be reduced (Meerlo et al., 1996c, 1997; Willner, 1993). Also, an increase in metabolic processes may be involved, reflected by an increase of body temperature during the circadian resting phase for several days after defeat (Meerlo et al., 1996a).

When individually and group-housed rats were exposed to sudden silence, both groups became highly immobile 2 days after defeat (Figure 2). However, upon repeated exposure, group-housed rats returned to their normal mobility after 7 days, whereas individually housed rats did not. Even after 21 days, individually housed rats were highly immobile, with immobility even increasing. Koolhaas et

al. (1990) also described extreme immobility in individually housed rats, lasting up to 10 weeks after defeat.

When rats were tested in an open field, individually housed rats were on average less active than group-housed rats (Figure 3). Although both groups became less active over time (probably because they became habituated to the repeated test), individually housed rats took on average significantly longer to leave their home base and moved significantly less in the outer zone. Latency periods for individually housed rats were significantly longer 21 days after defeat. They were also far less active in the outer zone of the open field 2 days after defeat compared to group-housed rats. However, differences in locomotion between the two groups were only significant at days 2 and 21 days after defeat. These findings agree with those of Meerlo et al. (1996b,c), reporting low levels of open field activity after social defeat in individually housed rats, being reduced when compared to isolated controls for a period of at least 7 days.

When rats were placed 14 days after defeat in an elevated plus-maze, validated for measuring anxiety (Pellow et al., 1985), those that were individually housed spent significantly less time on the open, unprotected arms of the maze than group-housed rats (Figure 4). This indicates that individually housed rats were more anxious than their group-housed counterparts. Similar levels of anxiety at 14 days after defeat were found in a previous study with individually housed rats (De Boer et al., unpublished data), in which defeated rats displayed a higher anxiety than their controls during 3 days following defeat.

Individually housing of rats after a social defeat also changed organ weights, measured 21 days after defeats (Table 1). It is well-established that stressful conditions enlarge the adrenals, diminish the thymus and spleen (Baldwin et al., 1995; Selye, 1950), and decrease the weight of reproductive organs (Selye, 1950). Although the spleen and testes of rats in our study did not differ in weight, the weight of the thymus and seminal vesicles of individually housed rats were significantly lower, and their adrenals were significantly larger.

When the DEX/CRF test was used, HPA activity was higher in individually housed rats at 21 days after defeat than in group-housed rats (Figure 5). In individually housed rats DEX was far less able to suppress the secretion of ACTH and corticosterone, and much more ACTH was released after CRF was administered. Moreover, in these rats, the AUC for the ACTH response tended to be higher. These results agree with those of Buwalda et al. (1999), who also showed a hyperactivity of the HPA-axis in defeated and isolated rats at 21 days after defeat. The high HPA activity in defeated and individually housed rats may be

explained by a reduced binding capacity of mineralocorticoid (MR) and glucocorticoid receptors (GR), as reported before by Sutanto et al. (1992), measured in the hippocampus 3 weeks after defeat. Human depression also coincides with increased HPA activity (Barden et al., 1995; Seckl et al., 1990). This may indicate that stress-related disorders in humans resemble those in rats following social defeat.

Implications for further research

Our results support the usefulness of the rat model using a social defeat as a stressor to study human psychopathologies, such as depression and anxiety (Koolhaas et al., 1997b). However, housing conditions seem to play an important role in the development of stress disorders in rats. Therefore the validity of the defeat model may be importantly increased by using housing conditions as one of the experimental variables. This will mimic different social settings, i.e. whether social support is available or not, which individuals may experience in everyday life.

Some factors that may affect our results were not within the scope of this study and should be mentioned. For instance, housing conditions before the stress treatments might influence comparisons between the two housing conditions (Brain and Benton, 1979). Furthermore, the degree of physical, olfactory, auditory, and visual contact with conspecifics, might influence the effect of isolation (Brain and Benton, 1979; Hurst et al., 1997). Also, characteristics of the social environment such as stability and quality of social relations are important determinants of social support (Seeman and McEwen, 1996). In addition, animals might be more or less vulnerable to pathological effects of stress, depending on the hierarchical position (social status) in a social group and on individual differences in coping style (Bohus et al., 1991; Fokkema et al., 1995; Koolhaas et al., 1997b; Mormède, 1990). Finally, the type of stress may determine whether social support will moderate the adverse effects of that stressor. For instance, after rats were exposed to a brief session of foot-shocks, group-housing did not moderate pathological effects (Van Dijken et al., 1992). This may be related to the non-social nature of foot-shock stress. Our contrasting results of a modulating role of type of housing in the detrimental effects of a social defeat, emphasizes that a single loss of social control differs from other, less naturalistic, stressors. Therefore, the social defeat model in rats may be more relevant for the biology of species, i.e. deals more with situations that organisms may meet in everyday life.

Chapter 4

Behavioural and physiological consequences of acute social defeat in growing gilts: effects of the social environment

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M.A.W. Ruis[1,2], J. de Groot[1,2], J.H.A. te Brake[1], E.D. Ekkel[1], J.A. van de Burgwal[1], J.H.F. Erkens[1], B. Engel[1], W.G. Buist[1], H.J. Blokhuis[1], J.M. Koolhaas[2]

[1] Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB Lelystad, The Netherlands
[2] Department of Animal Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

Abstract

Endocrine, behavioural and immunologic processes, together with body growth, were evaluated in gilts that were defeated at 10 weeks of age in resident-intruder tests. Immediately after defeat, gilts were either separated from or reunited with a familiar conspecific (litter-mate; always a barrow). Gilts were assigned to one of four treatments: (a) DI: defeat, followed by isolation (separation from original litter-mate; n = 8); (b) I: no defeat, isolation (control group; n = 9); (c) DP; defeat, followed by pair-housing (reunion with original litter-mate; n = 8); and (d) P: no defeat, pair-housing (control group; n = 8). The following general conclusions were derived: (1) social defeat caused pronounced short-term elevations in hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenal medullary activities, and of prolactin levels. Moreover, as soon as 1 hour after defeat, percentages of blood lymphocytes and neutrophilic granulocytes were, respectively, decreased and increased; (2) social defeat had some long-lasting influence on behaviour and physiology, but isolation predominantly determined responses in the longer term. Defeat, as well as isolation, resulted in increased cardiovascular activities compared to P controls, as observed in a novel object test (NOT: +7 days) and an aversion test (AVT: +14 days). Moreover, defeated as well as isolated gilts did not habituate to a repeated novel environment test (NET; -7, +2 and +7 days) in terms of frequencies of vocalizing, whereas P controls did. Isolation, through the separation from any other pig, was responsible for the other observed long-term characteristics, which developed progressively. Isolated gilts showed high mobilities and high cortisol responses in the repeated NET (+7 days), not being habituated. This contrasted the reactions of pair-housed gilts, which were much reduced. In addition to their high cardiovascular activities in the NOT and the AVT, isolated gilts also displayed higher heart rates in the repeated NET and during human presence following the NOT, compared to pair-housed gilts. Finally, isolated gilts were more inhibited to approach a novel object (in the NOT) than pair-housed pigs; and (3) stress responses of defeated gilts were modulated by the subsequent social environment. Stimulation of the HPA-axis (plasma- and salivary cortisol) was prolonged in those defeated gilts which were isolated (observed in the first hour). Changes in leucocyte subsets were still observed after 3 days in DI, but were 'normalized' within 1 day in DP gilts. Two days after defeat, habituation to the repeated NET in terms of mobility and salivary cortisol responses occurred in control and DP gilts, but not in DI gilts. We argue that these effects of the social environment shortly after defeat were related to a stress-reducing effect of a stable social relationship, i.e. social support.

Introduction

In modern pig husbandry, major welfare and production problems occur when pigs are regrouped and mixed with unfamiliar conspecifics. This practice, common with farmed pigs, leads to much aggression and vigorous fighting between strangers to establish a new social hierarchy of dominant and submissive animals (Meese and Ewbank, 1973). Most aggression occurs during the first few hours after mixing (Arey and Franklin, 1995; Meese and Ewbank, 1973; Olesen et al., 1996; Otten et al., 1997, 1999; Rushen, 1987; Stookey and Gonyou, 1994), with the establishment of a stable dominance hierarchy within 2 days (Friend et al., 1983; Meese and Ewbank, 1973; Olesen et al., 1996). In addition, chronically increased levels of less intense aggression for one (Stookey and Gonyou, 1994) or more weeks (Arey and Jamieson, 1997; Ekkel et al., 1997; Rushen, 1987) are observed.

The negative effects of mixing are often reflected in a reduction in body growth, which is not only seen within the first weeks after mixing (McGlone and Curtis, 1985; Stookey and Gonyou, 1994), but also over an extended period (months) (Ekkel et al., 1995b, 1996a; Lund et al., 1998). It is argued that a lower performance of a group depends on the inability of some individuals to cope with the new situation. There are indications that within pens of regrouped pigs, those animals showing a depressed growth, are also the ones which exhibit (social) defeat reactions (submissive behaviour) (Albinsson and Andersson, 1990). Instabilities of social relationships may lead to an aversive situation for submissive animals (persisting psychosocial pressure), while for dominants interactions are more predictable and can be controlled (Tuchscherer et al., 1998). On the other hand, acute social defeat may also have a long lasting negative impact on the loser animal, such as growth retardation, as shown in rodents (Koolhaas et al., 1997b; Miczek et al., 1990; Ruis et al., 1999). Defeated rodents display long term behavioural and physiological changes which much resemble symptoms of human psychopathologies, such as depression and anxiety. In pigs, Stookey and Gonyou (1994) demonstrated that a relatively short period of mixing (24 hours) may have a negative effect on body weight gain for up to 2 weeks thereafter.

Observations of defeated rodents indicate that the dynamics of stress responses of individual animals may be modulated by the social environment. Long lasting effects of acute social defeat were observed in singly housed rats, being greatly reduced in rats that returned to stable social groups of litter-mates (Ruis et al., 1999). The ability to have stable social relationships with conspecifics may protect individuals against the adverse effects of stress (i.e. social support; Sachser

et al., 1998). Studies in humans (Biondi and Picardi, 1996; Cohen, 1988; Coyne and Downey, 1991; Elmore, 1984; Miller and Surtees, 1995; Paykel, 1994) and nonhuman primates (Boccia et al., 1997) also show that social support permits individuals to cope more easily with stress factors. In pigs, it is not known whether social bonds with familiar pigs may enable animals to overcome social stress more easily, but Arnone and Dantzer (1980) suggest that an established social hierarchy protects pigs against behavioural and physiological consequences of emotional stimulation.

The present experiment reflects a fundamental design to gain more insight into social processes that occur in groups of pigs. Effects of social defeat and the importance of a (stable) social environment were investigated in pairs of pigs, for reasons of standardization. Endocrine, behavioural and immunologic changes were observed in growing gilts that were defeated in resident-intruder tests. Effects of the social environment following defeats were studied by either separation from (social isolation) or permitting attachment to (pair-housing) an original familiar companion (litter-mate). Control animals were not defeated and either were isolated or remained pair-housed. In the course of 3 weeks, body growth was measured and animals were exposed to four challenge tests classically reported to induce emotional arousal.

Materials and Methods

All procedures involving animal handling and testing were approved by the Animal Care and Use Committee of the Institute for Animal Science and Health (ID-Lelystad) in Lelystad, The Netherlands. Figure 1 shows the timing of experimental procedures.

Experimental housing and animals

The experiment was carried out in three successive and identical trials (batches). Two weeks before the expected dates of farrowing, in each trial, three multiparous sows (Great Yorkshire x Dutch Landrace) were transported from the experimental farm 'Bantham' in Maartensdijk to the experimental farm in Lelystad. Both farms are part of the Institute for Animal Science and Health (ID-Lelystad) and are located in The Netherlands. The experiment took place in three adjacent experimental rooms (each trial in another room). Piglets (Great Yorkshire x (Great Yorkshire x Dutch Landrace)) were born in farrowing pens (2.35 x 1.70 m) with partly slatted concrete floors. They were weighed and ear tattooed for identification

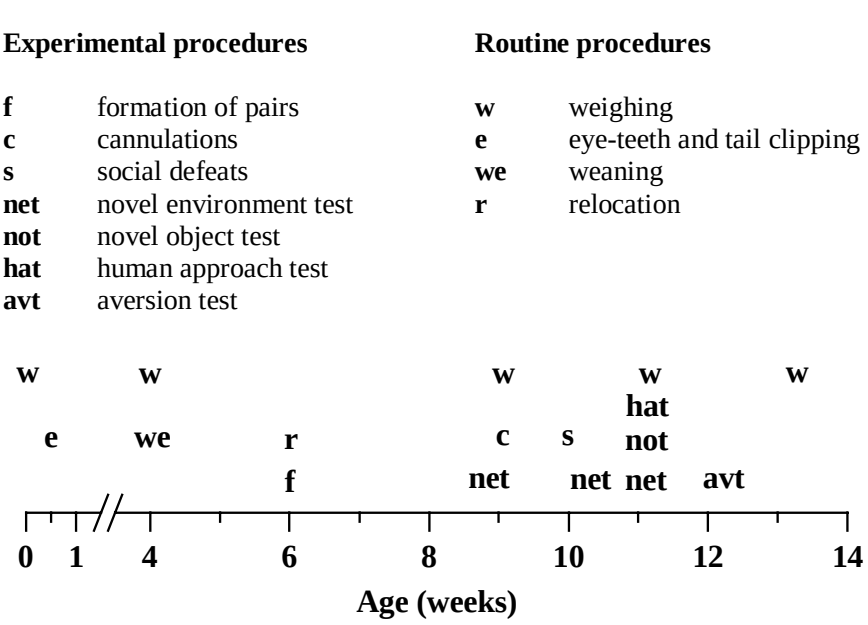

Figure 1. Timing of experimental and routine procedures.

within 1 day after birth. Castration of male piglets, eye-teeth and tail clipping, and iron injection, were carried out between 2 and 4 days of age. Piglets were weaned at 4 weeks of age. Within each trial, 10 to 12 pairs (litter-mates) of 6-week old animals were composed. Selected pigs were relocated but remained in the same room, while the other animals were removed. Pigs in each pair were weight matched and of both sexes. Whereas the barrow in each pair acted as a companion, the gilt was subjected to experimental treatments and observations, starting at 9 weeks and lasting until 13 weeks of age. Data were obtained from a total of 33 gilts. Pairs were housed in pens which measured 1.80 x 0.85 m, with partly slatted concrete floors and high (0.90 m) and solid wooden partitions between the pens preventing pigs from having visual and physical contact with other pigs. Experimental rooms were ventilated and temperature controlled, with temperatures kept between 22 ^{0}C (at birth) and 19 ^{0}C (at 14 weeks of age). Artificial lights were set on a 12 hour: 12 hour light regime with lights on at 06:00 h, and with no daylight visible in the rooms (total lux in the rooms during the light period ranging from 50 to 100). Food (commercial pelleted dry diets) and water (from nipple drinkers) were available ad libitum. From 6 weeks of age onwards, pigs were accustomed to handling by experimenters. To observe body growth during the experimental period, pigs were weighed at 9, 11 and 13 weeks of age.

Cannulations for repeated blood sampling

A total of 1 week before the social defeats, all experimental gilts received an indwelling catheter under complete anaesthesia. Pigs were food deprived for 12 hours, sedated with azaperone (2 ml i.m. per pig, Stresnil®, Janssen Pharmaceutica, Tilburg, The Netherlands) and anaesthetized with ketamine-HCL (10 ml i.m. per pig, Nimatek®, AUV, Cuijk, The Netherlands). A catheter was inserted through an ear vein into the jugular vein and the external part was attached to the ear, according to a technique described by Freriksen et al. (1996). Surgery of one pig took approximately 20 min, and before return to its home pen an additional 1 ml azaperone was injected. An operated pig was separated from the companion through partitions in the home pen for a few hours, until fully recovered. Pigs were treated with antibiotics (2 ml i.m. per pig, Ampicillan® 20%, AUV, Cuijk, The Netherlands) for 4 days to prevent infections. Between blood samplings, catheters were filled with sterile saline with heparin (500 IU/ml).

Social defeats and social environments

At 10 weeks of age, experimental gilts were removed from their companions and gently transferred to an adjacent testroom (between 8.00 and 12.00 h in the morning). In this testroom, previously selected 2-3 week older gilts were housed individually (already for a period of 2-3 weeks) in pens measuring 2.35 x 1.70 m. Previous selection of these gilts was based on behavioural resistance in a backtest. Only high resisting gilts were selected, thereby increasing the chance of choosing potentially aggressive animals (Ruis et al., 2000). Moreover, we selected only those gilts for the fights which were the winner in all of three encounters with nonexperimental pigs. In a resident-intruder paradigm, an experimental gilt (intruder) was individually introduced in the home pen of an aggressive gilt (resident), for a standard period of 15 min (see also De Jong et al, 2000). The start of fighting was subject to variation, but this period was sufficient to evoke vigorous and 'decisive' fighting. The fights involved behaviours such as parallel/inverse parallel pressings, head-to-head-knocks, head-to-body-knocks, head-tilts, bitings and displacements (Jensen, 1982; McGlone, 1985; Rushen and Pajor, 1987). An aggressive gilt was considered to be a winner when its opponent stopped fighting and started with defensive moves. At that time the winner was biting its opponent in the head region, particularly the ears (McGlone, 1985; Rushen and Pajor, 1987). By this submissive behaviour of the defeated pig further aggression was inhibited. All experimental gilts were defeated in the social encounters. None of the catheters was lost during the social confrontations.

Following defeats, experimental gilts were returned to their home pens and either separated from (isolation) or reunited with (pair-housed) the original companions (litter-mates). Separation took place by removal of the companion, shortly before the return of the gilt to its home pen. Control animals, which were not subjected to defeat, remained in their home pens and were either isolated (between 8.00 and 12.00 h in the morning; also by removal of the companion) or stayed together with their familiar conspecifics. Individuals and/or pairs were visually and physically separated. To summarize, the four treatments and numbers of animals assigned to the treatments were as follows: DI: defeat, followed by isolation (n = 8); I: no defeat, isolation (n = 9); DP: defeat, followed by pair-housing (n = 8); P: no defeat, pair-housing (n = 8).

Blood and saliva sampling

Blood samples of approximately 5 ml were withdrawn 5 min prior to and 5, 15, 30, 45 and 60 min after the social defeats. Due to loss of catheters, blood was only obtained from 7, 6, 7, and 6 gilts in the DI, I, DP and P groups, respectively. Samples were immediately transferred to polypropylene 10 ml centrifuge tubes containing EDTA (Vacuette®, Greiner B.V., The Netherlands) and placed on ice. Subsequently, the blood was centrifuged at 4 ^{0}C for 10 min at 2000g. Awaiting measurements of cortisol and catecholamines, half of each supernatant (approximately 1.5 ml) was stored at –20 ^{0}C. The other half was stored at –80 ^{0}C, awaiting ACTH and prolactin analyses. Hormone analyses were performed within 2 months after the collection of the plasma samples. Smaller amounts of blood (approximately 2 ml) were sampled at 3 days prior to, and also at 5 and 60 min, and 1 and 3 days after defeats. These samples were transferred to 5 ml evacuated centrifuge tubes containing heparin (Vacuette®, Greiner B.V., The Netherlands) and kept at room temperature. They were used within a few hours for leucocyte counts and differentiation of leucocyte subsets. By day 3, due to loss of catheters, blood could only be drawn from 6, 6, 6, and 5 gilts in the DI, I, DP, and P groups, respectively.

Saliva samples were taken simultaneously with blood samples, for determinations of (free) cortisol concentrations. Moreover, samples were taken during the (repeated) novel environment test (NET; see next section). This was done according to the same procedure as described before by Ruis et al. (1997). Briefly, saliva samples were collected by allowing animals to chew on two cotton buds simultaneously until the buds were thoroughly moistened. The buds were

placed in special centrifuge tubes and kept on ice until centrifuged for 5 min at 400 *g* to remove the saliva. Saliva was then stored at –20 ^{0}C until analysis.

Novel environment test (NET)

This test was performed at 7 days before, and 2 and 7 days after the social defeats. The novel environment consisted of an arena (3.8 x 3.0 m), with solid walls (1 m high) and a solid concrete floor (Figure 2). The arena was connected with a startbox (0.8 x 0.8 m) and both were separated by a sliding door. The arena was situated in a closed room without visible daylight, with total lux of 400 at 0.5 m above the floor. A camera was mounted on the ceiling above the arena and was connected to a video recorder and a monitor in a separate room.

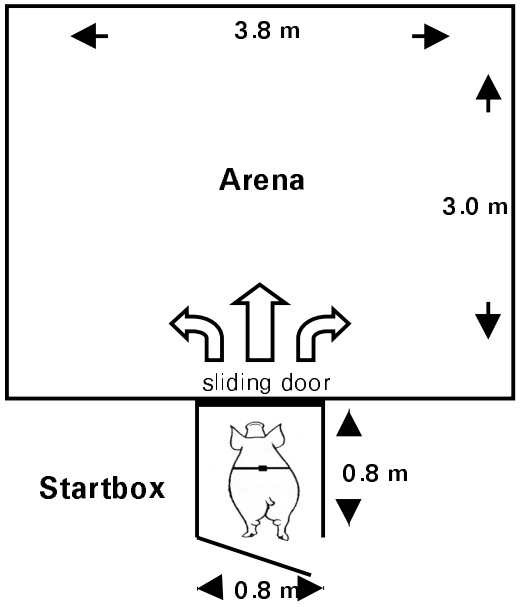

Figure 2. Lay out of the novel environment test (NET; see Materials and Methods).

After being removed from their home pens, individual gilts were gently driven into the startbox (through a corridor for 10-20 m). Immediately thereafter, a Polar® Sport Tester (PST; Polar Electro Oy, Kempele, Finland) was attached for recordings of heart rate (HR), according to a procedure described by Geverink et al. (1999). HR was recorded at 5 s intervals in beats per min (bpm). After 2 min, the door of the startbox was opened and the gilt was allowed to enter the arena. The gilt was then allowed to explore the arena for 10 min. The order of testing was randomized and the test location was cleaned between tests. All tests were carried out between 09.00 and 14.00 h. Behaviour was recorded on videotape and analyzed

afterwards with the software programme EthoVision® (Noldus Information Technology, Wageningen, The Netherlands). Behavioural parameters that were determined were the latency time to leave the startbox (all four legs outside the box) and the distance travelled in the arena (locomotion). Number of vocalizations in the arena were scored during the test. At the end of testing, the PST was removed and the gilt was gently driven back to the home pen. Time series of HR measurements for each animal were summarized by the following parameters: average HR in the startbox and the arena, (time-point of) peak HR upon opening of the startbox, HR increase (increase to the peak value), acceleration rate upon opening of the startbox (relative increase to the peak value: regression coefficient (RC)), and deceleration rate (relative decrease to pre-peak levels: RC). The cortisol response to the overall test (in test 3, including the novel object and human approach test: see next section) was determined by sampling saliva 5 min prior to and, 5 and 15 min after testing. Samples at t = -5 min were also used to follow baseline salivary cortisol concentrations.

Novel object (NOT) and human approach test (HAT)

At the end of the third novel environment test, 7 days after the defeats, a novel object was introduced during 2 min. The novel object consisted of a yellow and a grey bucket (tied together) and was lowered from the ceiling onto the floor of the arena and then lifted to a height of approximately 0.5 m above the floor. After 2 min, one experimenter suddenly entered the arena (sudden human approach) and remained in a stationary posture at one end of the arena for 1 min. The latency time of each pig to initiate contact with the novel object and with the experimenter was determined afterwards from videotape. During both procedures, vocalizations were scored directly. HR in both tests was summarized in similar parameters as for the novel environment test.

Aversion test (AVT)

With this test the degree of aversion of gilts was tested to a person wearing white overalls and having a syringe in one hand. Previously, antibiotic treatments were carried out following the cannulations (injections on 4 successive days). This was done by a person in white overalls. Since in all other situations, experimenters and farm personnel were wearing green overalls, gilts may predict, from the presence of the person in white, that they will be unpleasantly handled (aversion-learning; see Rushen, 1996). Two weeks after the defeat procedure, a gilt was provided with a PST in its home pen for HR monitoring, done by a person in

regular green overalls. Two min later, the person in white overalls entered the home pen, and remained in a stationary posture during 2 min. The syringe used for the antibiotic treatments was well visible, but was not used. Besides HR patterns (summarized in similar parameters as for the novel environment test), avoidance behaviour was directly recorded by scoring the latency time of the gilt to initiate contact with the handler, and total time contacting the latter.

Hormone and immunological determinations

Cortisol. Plasma cortisol was measured using a time-resolved fluoroimmunoassay (TR-FIA). The assay was performed as described for bovine plasma by Erkens et al. (1998), with the following modifications. Samples and steroid free plasma for the preparation of standards were diluted 1:10. Cortisol binding plasma proteins were inactivated at a temperature of 95 ^{0}C. A low and a high control sample and a 50/50% mixture were analyzed in all assays (n = 40) to check for linearity and reproducibility. Mean concentrations and inter-assay coefficients of variation (CVs) were 19.7, 62.8 and 43.9 ng/ml and 11.3, 6.2 and 6.9%, for low, high and 50/50% control samples, respectively. Intra-assay CVs (n = 14) of 4 samples with cortisol concentrations ranging between 20 and 70 ng/ml were 6.5±2.9% (mean±SD). Recovery of 20, 40, 80 and 160 ng of standard cortisol added per ml of three samples was 88±17, 96±12, 95±5 and 93±10%, for low, high and 50/50% control samples, respectively. The detection limit was 1.6 ng/ml. Salivary cortisol was measured in one assay on 1 day, by using a solid-phase radioimmunoassay (RIA) kit (Coat-A-Count Cortisol® TKCO, Diagnostic Products Corporation, Apeldoorn, The Netherlands), modified for pig salivary cortisol (Ruis et al., 1997). The detection limit of the assay was 0.13 ng/ml, and the intra-assay CV was 6.6%.

Plasma ACTH. A commercial ACTH assay® for human plasma (Nichols Institute Diagnostics, San Juan Capistrano, USA) was used according to the instructions of the manufacturer and was validated for porcine plasma. To avoid a decline of the samples ACTH concentration, that was observed for extended time intervals between thawing of the samples and start of the assay (but not for extended assay incubation), samples were thawed in series of 30, placed on ice and pipetted within 45 min, followed by the immediate start of the assay. Generally, assay incubation time at room temperature varied between 20 and 24 hours; for series within one assay the maximal difference in incubation time was 1 hour. Standards and samples (200 µl) were analyzed in duplicate. Radioactivity of the tubes was measured for 1 min in a model 1470 Wizard® gamma counter and data

were evaluated using Multicalc® software (Wallac Oy, Turku, Finland). A low and a high control sample and a 50/50% mixture were analyzed in all assays (n = 9). Mean concentrations and inter-assay CV's were 12.7, 152 and 78.2 pg/ml and 6.5, 3.2 and 2.9%, for low, high and 50/50% control samples, respectively. Intra-assay CV (n=14) of the mixed control sample was 3.9%. Recovery of 2.6, 7.75, 26.25 and 77.5 pg of ACTH (50 µl of kit standards E to H) added to 150 µl of the low control plasma was 114, 98, 105 and 96%, respectively. The detection limit was 1.0 pg/ml.

Plasma prolactin. For measurements of prolactin, a RIA was performed as reported by van Landeghem and van de Wiel (1978), with minor modifications, using an automated procedure as described by Erkens et al. (1992). Porcine prolactin (code PRL-IVO-19-1-79), with a potency of 1.07 towards AEW-SP-162-C, was used as standard and for iodination. Labelling buffer was 0.5M sodium phosphate, pH 7.4. Column elution and assay buffer was 0.05M sodium phosphate, 0.15M sodium chloride, 0.1% (w/v) sodium azide, pH 7.2, containing 1% (w/v) bovine serum albumin (BSA®, fraction V; Fluka AG, Buchs, Switzerland). One ml of a 1:8 dilution of donkey anti-rabbit solid phase (IDS, Boldon, England) with assay buffer, containing 0.1% (w/v) BSA, was used. Detection limit was 0.4 ng/ml. Intra- and interassay coefficients of variation were 10.7 (n = 12) and 9.0% (n = 5).

Plasma catecholamines. Plasma samples were analyzed for adrenaline and noradrenaline by high pressure liquid chromatography (HPLC) with electrochemical detection following a liquid extraction (twice) according to the procedure described by Smedes et. al. (1982). From each sample, a volume of 100 µl was used. DHBA (3,4-dihydroxybenzylamine) was used as an internal standard. The HPLC system consisted of an autosampler (Perkin Elmer ISS-101®, Perkin Elmer, Norwalk, Connecticut, USA), a Perkin Elmer 410® HPLC pump (Perkin Elmer, Norwalk, Connecticut, USA), a vacuum degasser (X-Act®, Jour Research, Onsala, Sweden), an EC-controller (INTRO®, Antec Leyden, Leiden, The Netherlands) with inbuilt columnoven, pulsdamper (SSI®, Antec Leyden, Leiden, The Netherlands) and detector cell (VT-03®, Antec Leyden, Leiden, The Netherlands). The potential was set at 0.611 V (versus Ag/AgCl in 2 mM [Cl^-]) using the in situ Ag/AgCl reference electrode (ISAAC®, Antec Leyden, Leiden, The Netherlands). The catecholamines were separated using a Nucleosil® C18 column (length 25 cm, i.d. 4 mm, particle size 5 µm; Macherey Nagel, Düren, Germany). The mobile phase consisted of 72 mM citric acid monohydrate, 42 mM $Na_2HPO_4.2H_2O$, 1.1 mM 1-octanesulfonic acid (sodium salt), 0.4 mM EDTA, 2.0 mM NaCl, 3 % acetonitril. The pump was operated at a rate of 1 ml/min. The

column, pulsdampener and detectorcell were maintained at a constant temperature of 30^0C. The detector signal was recorded by a chartrecorder (BD112®, Kipp & Zn, Delft, The Netherlands). The detection limit (defined as the amount of compound producing a peak twice the basal noise) was typically 0.6 pg for noradrenaline and 1.0 pg for adrenaline.

White blood cell counts and differentiation. Total leucocyte counts were determined by means of an automatic cell counter (Sysmex®, F-800, TOA Medical Electronics, Kobe, Japan). White blood cells were differentiated in lymphocytes and monocytes/granulocytes by adding quicklyser II® (TOA Medical Electronics, Kobe, Japan). To control for accuracy of this method, blood smears were stained with a Hema-Tek slide-stainer (modified Giemsa). A total of 100 cells was counted microscopically, in which the lymphocytes and monocytes, and neutrophilic, eosinophilic and basophilic granulocytes were differentiated. Only 1-5% of leucocytes were eosinophilic and basophilic granulocytes, and monocytes. Therefore, the %lymphocytes and monocytes/granulocytes from the cell counter quite accurately represent the %lymphocytes and %neutrophilic granulocytes, and these were used for statistical analysis.

Statistical analysis

A mixed analysis of variance model was used to analyze data with respect to variables in the NOT, HAT, and AVT, and the development of weight. Effects for litters and animals within litters were entered as random effects in the model. These random effects account for possible correlation between litter-mates and correlation between observations on the same animal within a litter. Effects for social defeat (yes or no), housing (isolation or pairs), and trial (1 to 3) were entered as fixed effects in the model. The model included main effects and the interaction between social defeat and housing. In addition, for variables in the repeated NET, time relative to the (start of) treatments (-7, 2 and 7 days) was included as a fixed effect in the model. When no significant interaction was found, the model was reduced to main effects only. Data on latency times in the NET showed heterogeneity and were therefore logarithmically transformed prior to analysis. For this parameter, treatment effects were expressed by multiplicative factors between treatment means. Components of variance were estimated by restricted maximum likelihood (REML; Engel, 1990). Treatment effects (main effects and interactions) were tested with the Wald test (Buist and Engel, 1994). Acute hormone responses and changes in leucocyte subsets were analyzed by summary statistics to circumvent the complicated dependence structures of closely spaced time-points.

For these variables, a paired t-test was used to test changes from baseline values. Moreover, analysis of variance was performed for treatment effects within separate time-points. In case of significant results, comparisons between groups were made using t-tests with a pooled variance estimator. All calculations were performed with the statistical programming package Genstat 5® (1993). Effects were considered significant if $p<0.05$. Unless stated otherwise, data are presented as mean(±SEM).

Results

HPA-axis activity

Compared to levels prior to stress procedures, social defeat caused significant increases ($p<0.01$) in ACTH and (plasma and salivary) cortisol concentrations, with peak values generally at t = 5 min (Figure 3). Undefeated controls either showed no (P gilts) or moderate (I gilts; elevated salivary cortisol concentration at t = 5 min: $p<0.05$) hormone responses. Although not found for ACTH levels, the decline of cortisol concentrations to baseline values (recovery) following social defeat differed according to the way of housing. While (plasma and salivary) cortisol concentrations were recovered after t = 30 min in DP gilts, cortisol concentrations remained significantly ($p<0.05$) elevated during the 60-min sampling period in DI animals. This effect of housing conditions following defeat was substantiated by comparisons between groups. At the end of the 60-min sampling period there was a significant interaction between the factors housing and defeat for plasma- ($p<0.05$) and salivary cortisol ($p<0.05$) concentrations. At this time, only those gilts which were isolated following defeat showed higher plasma- and salivary cortisol concentrations, compared to both control groups ($p<0.05$) and P controls ($p<0.05$), respectively.

Baseline salivary cortisol concentrations, obtained from t = -5 min samplings of saliva in the repeated novel environment test, did not change in the course of 1 week following the (start of) treatments. Concentrations were 0.96±0.12, 0.81±0.08, 0.87±0.09, and 0.89±0.10 ng/ml at t = –7 days, 0.84±0.11, 0.89±0.13, 0.79±0.14, and 0.76±0.09 ng/ml at t = +2 days, and 0.90±0.10, 0.82±0.13, 0.74±0.14, and 0.71±0.13 ng/ml at t = +7 days, respectively, for DI, I, DP and P gilts.

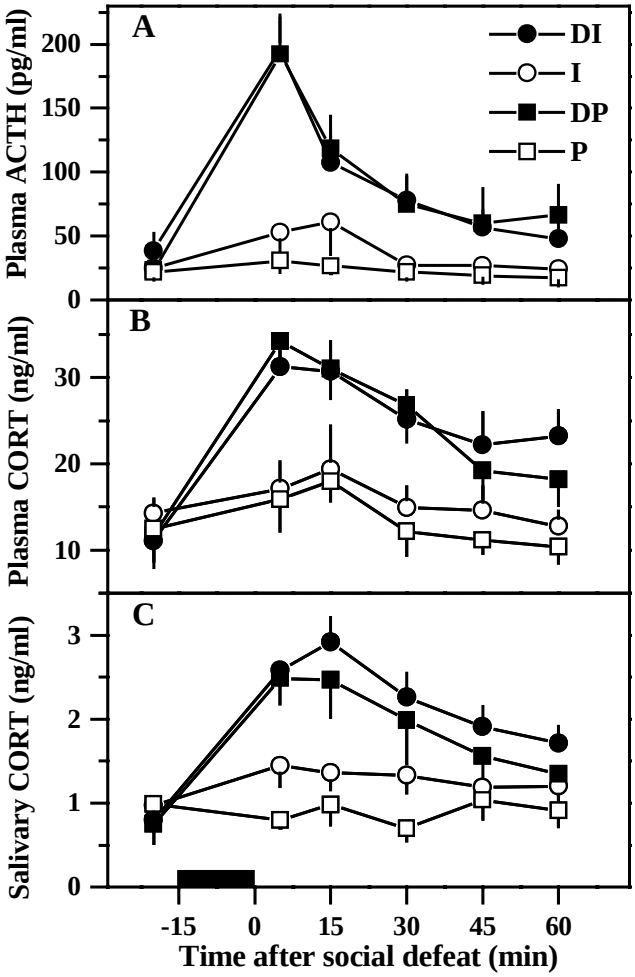

Figure 3. Mean (±SEM) concentrations of plasma ACTH (A) and cortisol (CORT) in plasma (B) and saliva (C) in the course of 1 hour following the (start of) treatments. DI, defeat, isolation (sample size: n = 7); I, isolation (controls; sample size: n = 6); DP, defeat, pair-housing (sample size: n = 7); P, pair-housing (controls; sample size: n = 6). Horizontal black bar indicates the confrontation period. For significant changes within and differences between treatments, see Results.

Plasma catecholamine concentrations

Figure 4 shows that social defeat significantly increased plasma catecholamine concentrations, both compared to baseline values (p<0.05 at least), and to control treatments (main effect for defeat: p<0.05). Peak values were observed at 5 min following the end of the fights, and concentrations were recovered thereafter. In undefeated controls, no significant increases in catecholamine concentrations were observed. Interestingly, at t = 60 min,

noradrenaline levels in isolated gilts were higher than in pair-housed animals (significant main effect for housing: $p<0.05$).

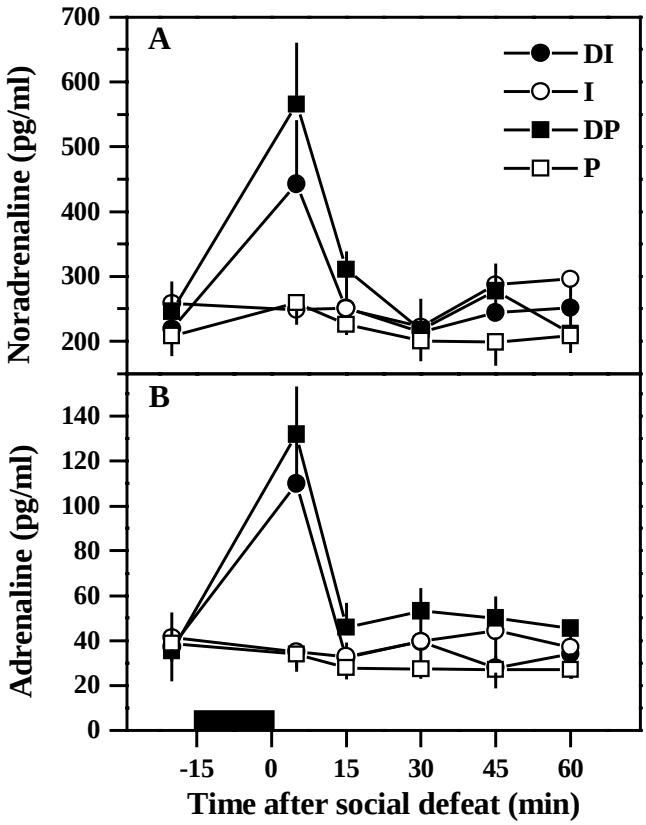

Figure 4. Mean (±SEM) plasma noradrenaline (A) and adrenaline (B) concentrations in the course of 1 hour following the (start of) treatments. DI, defeat, isolation (sample size: n = 7); I, isolation (controls; sample size: n = 6); DP, defeat, pair-housing (sample size: n = 7); P, pair-housing (controls; sample size: n = 6). Horizontal black bar indicates the confrontation period. For significant changes within and differences between treatments, see Results.

Plasma prolactin concentrations

Following social defeat, prolactin concentrations were significantly elevated at t = 5 min, but not thereafter, both compared to baseline values ($p<0.05$) and to control treatments (main effect for defeat: $p<0.05$) (Figure 5).

Changes in peripheral blood leucocytes

Figure 6 shows the changes in percentages of leucocyte subsets in the four treatment groups. The changes are presented relative to baseline values (presented as the 0-line in the figure). In both control groups, no significant changes from

baseline values in percentages of leucocyte subsets were observed. However, as soon as 1 hour after defeat, defeat stress resulted in significant lowerance in percentage of lymphocytes, compared to baseline values ($p<0.05$) and to those of control groups (main effect for defeat: $p<0.05$). Simultaneously, percentages of neutrophilic granulocytes were significantly enhanced, compared to baseline ($p<0.05$) and control (main effect for defeat: $p<0.05$) values. On days 1 and 3, values were returned to baseline and to those of controls in DP, but not in DI gilts. On days 1 and 3, in DI gilts there were still tendencies for a lowered lymphocyte percentage ($p=0.06$ and $p=0.08$, respectively) and for an enhanced neutrophilic granulocyte percentage (both $p=0.07$). Moreover, at these time-points, between-group comparisons showed significant ($p<0.05$ at least) interaction effects between the factors defeat and housing. In DI gilts, lymphocyte percentages were significantly decreased ($p<0.05$ at least) compared to those of both control groups. The increase in granulocyte percentages in DI gilts was only significant ($p<0.05$ at least) compared to those of P controls.

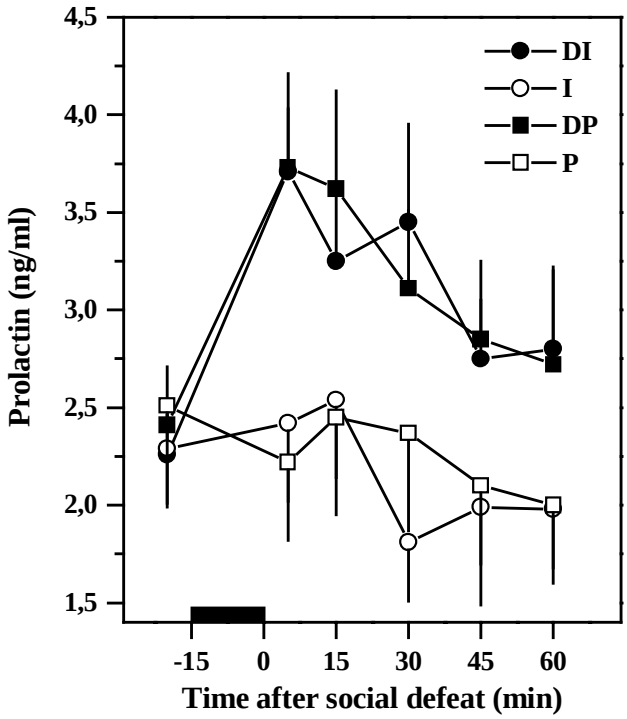

Figure 5. Mean (±SEM) plasma prolactin concentrations in the course of 1 hour following the (start of) treatments. DI, defeat, isolation (sample size: n = 7); I, isolation (controls; sample size: n = 6); DP, defeat, pair-housing (sample size: n = 7); P, pair-housing (controls; sample size: n = 6). Horizontal black bar indicates the confrontation period. For significant changes within and differences between treatments, see Results.

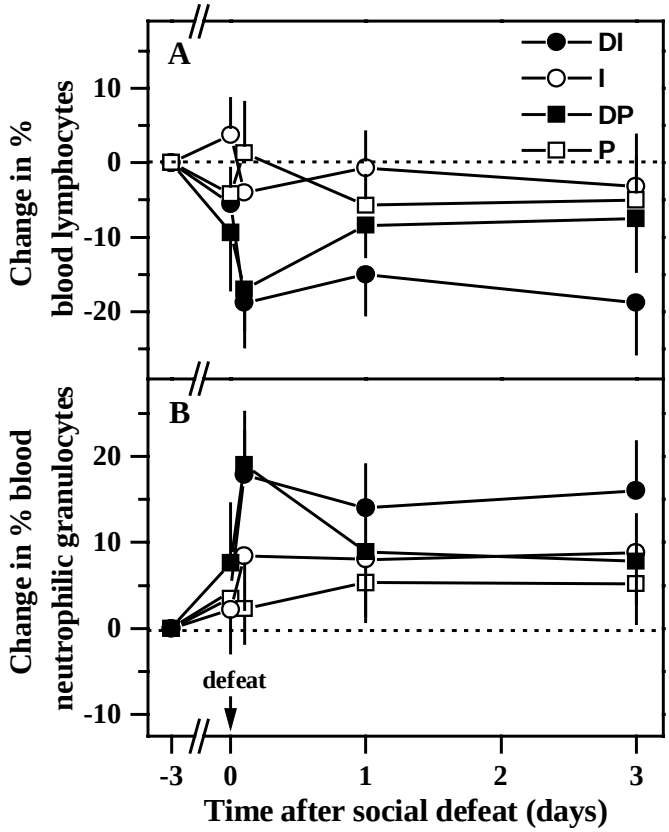

Figure 6. Mean (±SEM) changes in percentages of blood lymphocytes (A) and blood neutrophilic granulocytes (B) in the course of 3 days following the (start of) treatments. The changes are presented relative to baseline values (0-line). DI, defeat, isolation (sample size: n = 6); I, isolation (controls; sample size: n = 6); DP, defeat, pair-housing (sample size: n = 6); P, pair-housing (controls; sample size: n = 5). For significant changes within and differences between treatments, see Results.

Responses to the repeated novel environment test (NET)

Table 1 shows the behavioural characteristics and cortisol responses of gilts to the repeated NET. HR patterns during the tests are shown in Figure 7.

Latency times to enter the arena. No differences were observed between test groups, but following the first exposure to the NET, latency times to leave the startbox were much reduced (about a factor 3.4±0.5) in the subsequent tests (significant main effect for the factor time: $p<0.01$).

Locomotions. Changes in locomotions depended on defeat and housing procedures, and were characteristic for each test (three-factor interaction: $p<0.05$). While at 2 days after the (start of) treatments mobility was reduced in most animals when compared to the first test (being significant in DP gilts; $p<0.05$), DI gilts

were still highly mobile. A comparison of groups showed a significantly (p<0.05) higher mobility for DI gilts than for DP animals. At 1 week after the (start of) treatments, both DI and I gilts showed high mobilities, whereas locomotion had declined significantly (p<0.05) in both groups of pair-housed gilts when compared to the first test. Within this time-point, no significant differences were observed between the treatment groups.

Table 1. Behavioural and cortisol responses (mean±SEM) to the repeated novel environment test (NET).

	Test at (days)	DI	I	DP	P
Latency time	-7	31.6±18.3	31.3±11.7	18.5±11.5	24.5±13.8
(sec)	2	5.9±1.7**	7.1±1.5***	5.0±1.3**	5.6±0.8**
	7	5.0±1.3**	4.8±1.1***	4.8±0.6**	4.9±0.8**
Locomotion	-7	100±7	102±12	109±6	107±3
(m)	2	102±6^{A}	88±11^{A,B}	84±9^{**,B}	96±8^{A,B}
	7	100±11	98±6	90±6**	80±13**
Vocalizations	-7	109±20	96±20	106±15	114±18
(number)	2	141±13	133±21	146±12**	122±20
	7	121±18^{A,B}	123±20^{A,B}	141±25^{A}	83±14^{**,B}
CORT response	-7	1.83±0.33	2.03±0.26	2.13±0.37	1.87±0.25
(ng/ml)	2	1.63±0.13	1.58±0.25**	1.49±0.24**	1.30±0.17**
	7	1.83±0.18^{A}	1.77±0.21^{A}	1.24±0.16^{***,B}	1.42±0.11^{**,B}

DI, defeat, isolation (n = 8); I, isolation (controls; n = 9); DP, defeat, pair-housing (n = 8); P, pair-housing (controls; n = 8). ^{A,B}Means with different superscripts within the same row differ (p<0.05). Asterisks (*) indicate changes within groups (vertically), compared to values in the first test (at –7 days): $^{*}p$<0.1, $^{**}p$<0.05, $^{***}p$<0.01. Interaction and main effects are mentioned under Results.

Vocalizations. There was a tendency for an interaction (p=0.06) between the factors defeat and housing for the change in numbers of vocalizations. Two days after the (start of) treatments, most animals except P controls vocalized more often than they did in the first test, being significant (p<0.05) for DP gilts. One

week after the (start of) treatments, P controls vocalized significantly ($p<0.05$) less, whereas in the other groups numbers of vocalizations did not differ from those in the first test. Within this time-point, DP gilts vocalized significantly ($p<0.05$) more than P controls.

Cortisol responses. For this parameter, the three-factor interaction was significant ($p<0.05$) and patterns of changes followed those for locomotions. DI gilts at 2 days, and DI and I gilts at 7 days after the (start of) treatments, showed cortisol responses as high as in the first test. Responses in the other groups, however, were significantly ($p<0.05$) reduced at these time-points. One week after the (start of) treatments, cortisol responses of isolated (DI and I) gilts were significantly ($p<0.05$) higher than those of pair-housed (DP and P) animals.

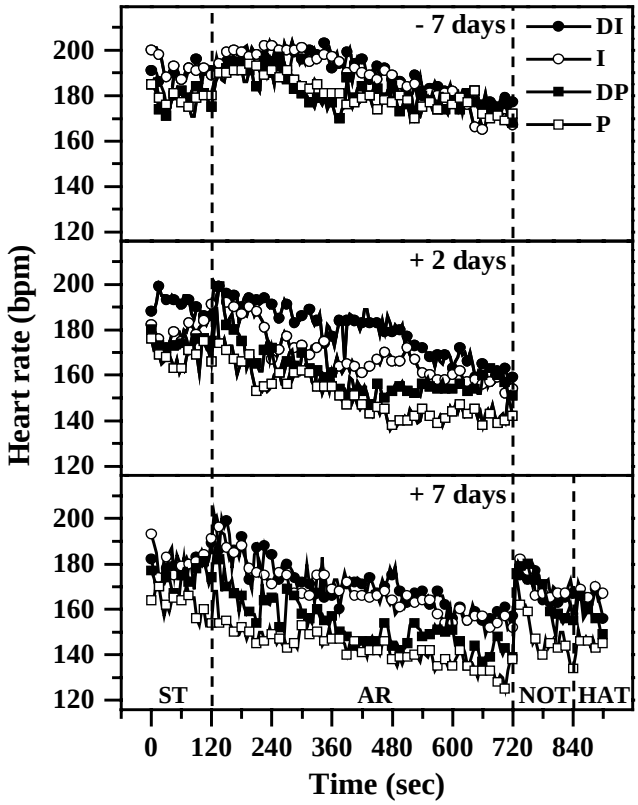

Figure 7. Mean heart rates (HRs) during the repeated novel environment test (NET) at 7 days before (upper panel), 2 days after (middle panel) and 7 days after the (start of) treatments (lower panel). Every third data point is shown. The latter test was inclusive the novel object test (NOT) and human approach test (HAT). For a detailed description, see Materials and Methods. ST: startbox; AR: arena. DI, defeat, isolation (n = 8); I, isolation (controls; n = 9); DP, defeat, pair-housing (n = 8); P, pair-housing (controls; n = 8). For significant changes within and differences between treatments, see Results.

Heart rate (HR). HR decreased in all animals with repeated exposure to the test (Figure 7). This was shown by significant (p<0.01 at least) main effects for the factor time (relative to the (start of) treatments) for average HR in the startbox (185.0±3.7, 179.4±3.9, and 175.3±3.6 bpm) and in the arena (185.7±3.2, 164.6±3.4, and 158.3±3.2 bpm), at -7, 2 and 7 days, respectively. However, HR patterns diverged according to experimental procedures, with housing conditions as the predominant factor. Overall, the average HR in the startbox tended (p=0.09) to be higher in isolated than in pair-housed gilts: 182.0±4.2 and 176.7±3.2 bpm, respectively. Isolated animals also displayed higher HR in the arena. Here, a significant (p<0.001) interaction between the factors housing and time was observed. Whereas the average HR was 185.7±3.2 bpm before the (start of) treatments, average HR for isolated and pair-housed gilts were, respectively, 172.3±4.4 and 157.0±4.2 bpm at 2 days (within-test comparison: significant difference, p<0.05), and 167.8±4.0 and 148.8±4.1 at 7 days after the (start of) treatments (within-test comparison: significant difference, p<0.001). For responses to the opening of the startbox, there was a significant (p<0.01) interaction between the factors time and housing for peak HR. By day 7, the difference between isolated and pair-housed gilts was significant (p<0.01): 208.0±5.6 and 186.6±5.8 bpm, respectively. No significant effects were observed for time-points of peak HR, HR increases, and HR acceleration and deceleration rates.

Responses to the novel object (NOT) and human approach test (HAT)

Latencies to initiate contact. At 7 days after the (start of) treatments, isolated gilts, irrespective of previous social defeat, took significantly longer to contact the novel object than those that were pair-housed (significant main effect for the factor housing, p<0.01). Latency times were 39.6±8.6 and 19.7±3.2 s for individually and pair-housed gilts, respectively. Upon sudden entrance of an experimenter in the arena, latency times to contact the latter did not differ between the experimental groups.

Vocalizations. There were no differences in numbers of vocalizations between the experimental groups, neither in response to the novel object, nor in response to the sudden approach of an experimenter. Frequencies of vocalizing were 27±11, 32±14, 35±12, and 23±7 in the NOT, and 20±6, 25±10, 27±9, and 16±7 in the HAT, respectively, for DI, I, DP and P gilts.

Heart rate (HR). For responses to the novel object, HR acceleration rates and peak HR did not differ between groups, but increases in HR were significantly (main effect for housing: p<0.05) higher in pair-housed (48.2±5.3 bpm) than in

isolated animals (35.6±5.2 bpm) (see the lower panel of Figure 7). Animals being defeated showed significantly (main effect for the factor defeat: $p<0.01$) lowered HR deceleration rates (but their acceleration rates did not differ). RCs of HR deceleration rates for defeated and non-defeated gilts were, respectively, -0.12±0.1 and -0.50±0.1. However, during the overall test HR was significantly ($p<0.05$ at least) lower in the P gilts than in gilts of the other groups; average HRs were 168.8±7.4, 169.9±6.8, 164.9±5.5, and 148.5±5.5 bpm, in DI, I and DP and P gilts, respectively (interaction not significant ($p=0.10$)).

Responses to the human approach were characterized by higher peak HR in isolated animals (178.5±4.9 bpm) compared to that in pair-housed ones (164.8±5.0 bpm) (significant main effect for housing: $p<0.05$) (see the lower panel of Figure 7). HR increases, and HR acceleration and deceleration rates, did not differ between groups. Average HR during the presence of the experimenter was higher in isolated (165.2±4.5 bpm) than in pair-housed animals (151.7±4.6 bpm) (significant main effect for housing: $p<0.05$).

Responses to the aversion test (AVT)

Latencies to contact experimenter. Latency times to contact an experimenter dressed in white overalls and total time contacting the latter did not differ between treatments. Latency times were 12.5±5.1, 16.0±8.2, 9.9±6.7 and 7.3±4.9 s, and total times of contact were 83.5±18.3, 92.2±12.4, 98.8±15.6 and 102.4±15.0 s, for DI, I, DP and P gilts, respectively.

Heart rate (HR). For average HR during the 2 min prior to the entrance of the person in white overalls, a tendency ($p=0.07$) for an interaction effect was observed between the factors housing and defeat (Figure 8). Compared to other treatment groups, average HR in P controls was lower, being significantly ($p<0.05$) lower than in I controls. The interaction was significant ($p<0.05$) for the average HR during the 2 min exposure to the experimenter in white overalls. Again, P controls showed lower HR values, being significantly ($p<0.05$ at least) lower compared to all other groups. Peak HRs were affected by housing conditions (significant main effect: $p<0.05$), and were 171.5±2.9 and 161.2±3.2 bpm, in isolated and pair-housed gilts, respectively.

Body growth

Body weights did not differ between treatment groups at 1 week before the (start of) treatments (21.7±1.7, 22.9±2.1, 21.3±1.8, and 23.2±2.2 kg), 1 week thereafter (36.0±2.0, 35.9±2.2, 34.9±2.1, and 37.3±2.8 kg), and 3 weeks thereafter

(47.0±2.8, 46.6±2.8, 45.4±2.5, and 47.8±3.5 kg), respectively, for DI, I, DP, and P gilts.

Discussion

We will discuss our findings by raising three issues. First, we describe the severe acute consequences of social defeat, causing strong endocrine and immunological changes in the loser animal. Secondly, we discuss the behavioural and physiological effects of acute social defeat, and of social isolation, in the longer term. Finally, some indications are given for the occurrence of social support following social defeat.

(1) Socially defeated gilts show strong acute endocrine changes, as shown by elevations in hypothalamic-pituitary-adrenal (HPA) activity, sympathetic-adrenal medullary activity, and prolactin levels. Elevations in HPA- and sympathetic-adrenal medullary activities during agonistic interactions have been reported before in the pig (Otten et al., 1997, 1999), and in other species (Sachser et al., 1998; Von Holst, 1985).

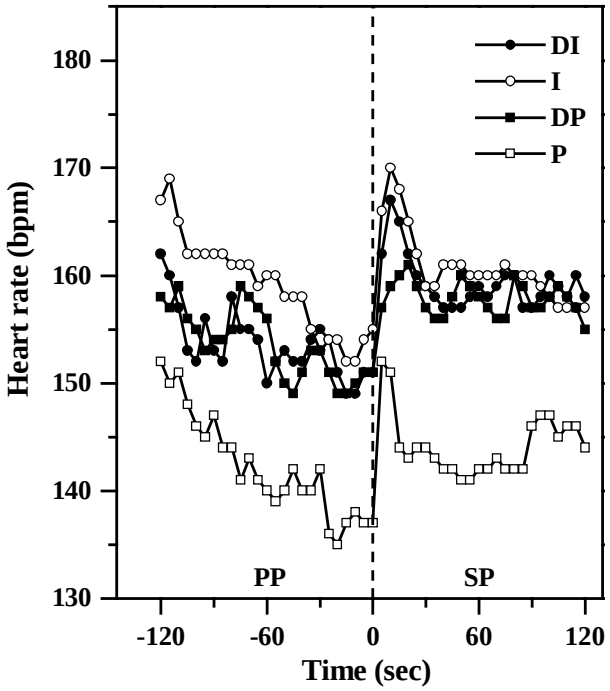

Figure 8. Mean heart rates (HRs) in the aversion test (AVT) at 2 weeks after the (start of) treatments. At t = 0 a person in white overalls entered the home pen. PP: pre-stimulation period; SP: stimulation period. DI, defeat, isolation (n = 8); I, isolation (controls; n = 9); DP, defeat, pair-housing (n = 8); P, pair-housing (controls; n = 8). For significant changes within and differences between treatments, see Results.

Most pronounced endocrine changes have been found to occur in those animals being defeated (Marchant et al., 1995; Sachser, 1987). The hormonal changes measured in this study were generally short-lived following termination of the acute social stress, which was most pronounced for plasma catecholamine levels. In fact, catecholamine levels might already be much decreased at the first sampling point after the fighting period. This may also explain the relatively low peak levels, compared to, for instance, those reported by Otten et al. (1997, 1999). Little is known about the effects of social stress on plasma prolactin levels in pigs, but in rats it was shown that prolactin release is augmented under conditions of fear (Meerlo et al., 1999; Zou et al., 1998). When extrapolated to pigs, this may substantiate that the loser pigs are highly fearful during encounters. This fearfulness may be related to the unpredictability and uncontrollability of the situation, which are factors held responsible for increments in prolactin (Muir and Pfister, 1987). Consequently, measurements of prolactin may provide a valuable tool for stress assessments in pigs. Our data also demonstrate defeat-induced changes in blood cellular immunological characteristics, by shifts in percentages of leucocyte subsets. While percentage of lymphocytes was decreased (lymphopenia), that of neutrophilic granulocytes was increased (granulocytosis), visible as soon as 1 hour after the encounters. Similar changes in circulating leucocyte subsets have been found in socially stressed rats (Stefanski and Engler, 1998) and chickens (Gross and Siegel, 1983), and in pigs being shipped (McGlone et al., 1993b). It is likely that endocrine factors released during stress, modulate leucocyte trafficking, which results in a redistribution of leucocytes between blood and other immune compartments (Dhabhar et al., 1995). Corticosteroids seem to be the major mediators of this effect (Dhabhar et al., 1995; Wallgren et al., 1994).

(2) Some long-term behavioural and physiological changes in defeated gilts may represent long lasting effects of the acute defeat stress, but social isolation predominantly determined the responses of gilts in the course of time. Neither social defeat, nor social isolation, did affect body growth and baseline cortisol concentrations in the course of time. However, some characteristics of defeated gilts, also observed for isolated gilts, involved increases in cardiovascular activity and vocal responsivity. In reaction to certain stimuli (NOT, AVT), higher cardiovascular activities were maintained in defeated as well as in isolated gilts, compared to those of pair-housed control gilts. Although we cannot entirely distinguish between physical and emotional causes of these cardiovascular activities, we suggest that at least some emotional arousal was involved. Gross indices of locomotory behaviour did not differ between, for example, defeated and

undefeated gilts (no differences in inhibitory (latency) behaviours). Both the NOT (Hopster et al., 1999) and tests which resemble the AVT (Rushen, 1996) are considered to be fear-eliciting challenges, and higher levels of fear in defeated as well as isolated gilts may therefore also maintain higher HRs in these animals. Surprisingly, we also measured high HRs during the two-min pre-exposure period in the AVT. Because our HR measuring technique may induce some disturbance, we suggest that this reflected a higher cardiac responsiveness of defeated and isolated gilts, rather than higher resting (baseline) HR values of these gilts in their home environment. Consequently, it may be suggested that even minor challenges elicit fear-related HR responses in defeated as well as in isolated animals. Another characteristic of defeated as well as isolated gilts was that they did not show habituation to the repeated NET with respect to the number of vocalizations, while pair-housed controls did so. Vocalizing has been shown to be positively correlated to locomotory behaviour and adrenocortical responses (Désautes et al., 1997). However, the present study does not provide evidence for such a relationship, since patterns of vocalizations did not necessarily parallel those of locomotions and cortisol responses. We believe that different emotions, i.e. social motivation (search for social contact) and general fearfulness, are contributing to vocal responsiveness.

Social isolation predominantly determined behavioural and physiological characteristics of gilts in the longer term. The effects of social isolation developed progressively which was shown by the absence of (pronounced) acute hormonal and immunological responses to this procedure. Staying in its home environment may be responsible for this initial non-responsiveness to isolation. This is supported by Puppe et al. (1997), who reported that piglets appear to have more problems in coping with a new housing environment than with social disturbances. In our study, after 1 hour of isolation, increments in noradrenaline levels (relative to pair-housed animals) occurred. Houpt et al. (1988) and Parrott et al. (1994) also found that isolation induced noradrenaline release. An increase in physical activity may mainly be responsible for this (Goldstein, 1987), since isolation in a known environment was observed to increase explorative behaviour (Carbonaro et al., 1992; Poindron et al., 1994). This suggests that social motivation underlies this behaviour, as a search for interactions with conspecifics. As argued by Jensen et al. (1999), animals deprived of social stimuli in the home environment may also be more responsive to novel environmental stimuli. Testing of isolated control gilts in the repeated NET showed that they initially (at 2 days) habituated well in terms of locomotory and adrenocortical responses, but less habituation was observed at 7

days. Regarding these reactions, pair-housed gilts habituated well to the repeated tests at 2 and 7 days. In pigs, Von Borell and Ladewig (1992) interpreted increased locomotory activities and greater adrenocortical responsiveness in a novel environment (being positively related like in the present study) in terms of excitability. This excitability may represent components of emotional arousal such as fear and/or exploratory motivation, known to occur in tests involving novelty (Boissy and Bouissou, 1995), such as the NET. We have repeated the NET and consequently expected that the novelty of the test decreased. When the test becomes less frightening, then persistence of, for example, high locomotory behaviour rather reflects exploratory motivation than fear. Alternatively, a higher emotional arousal may slow down habituation-like processes, and therefore fearfulness towards the challenge may be maintained. Thus, reactions to the NET of isolated gilts are not easily explained in terms of specific emotions, but their responses to the NOT suggest that the animals are more fearful. Regardless of previous social defeat, they were more inhibited to approach the novel object, which is a validated measure of fear in cows (Hopster et al., 1999). Besides the already mentioned cardiovascular characteristics of isolated gilts, isolated gilts also displayed higher HRs than pair-housed gilts in the startbox and the arena of the repeated NET, with the most pronounced difference at 7 days. Whereas the higher HR of isolates in the arena may be related to their higher amount of activity, a primarily emotional response, i.e fear, may determine their HR response in the startbox, in which animals were more or less restricted to move. Additionally, differences in average HR between animals under the respective housing conditions existed in the HAT, but in this test it was more difficult to discriminate between physical and emotional causes. Peak HRs in isolated gilts were found to be higher in several tests, except the NOT, presumably related to higher HR levels prior to the latter stimulus.

To conclude, socially defeated, and to a greater extent socially isolated gilts, seemed to be more responsive to changes in their environment (sensitization process: Koolhaas et al., 1997b; Post, 1992). We have raised some points here for an increased emotional reactivity of defeated gilts, but body growth as an important indicator of performance, was not affected. This contrasts findings in rodents, in which acute social defeat causes suppression of body growth for several weeks (Koolhaas et al., 1997b; Miczek et al., 1990; Ruis et al., 1999). In pigs, however, depressed body growth is observed following mixing (Ekkel et al., 1995b, 1996a; Lund et al., 1998; McGlone and Curtis, 1985; Stookey and Gonyou, 1994), which may substantiate that a lower performance is not (only) related to short-term

vigorous fighting, but to a prolonged coexistence of dominant and submissive animals. For the loser pigs, the stress of being defeated together with the continuing stress of threats and submission may be primarily responsible for a reduction in performance. This is supported by Stookey and Gonyou (1994), although the same authors also suggest that a relatively short period of mixing, that is 1 day, partly attributes to a long lasting (up to 2 weeks thereafter) setback in body weight gain. Finally, increases in emotional arousal and decreases in habituative abilities of socially isolated gilts, may emphasize that pigs deprived from any social contact, which is a rather unnatural situation, are less able to cope with environmental stimuli than those animals which are socially housed.

(3) Some characteristics of stress responses to social defeat were indicative for a modulating effect of the subsequent social environment. Although not reflected in catecholamine and prolactin response profiles, stimulation of the HPA-axis (plasma- and salivary cortisol) was prolonged in those defeated gilts which were isolated (observed in the first hour). Measures of circulating ACTH, however, did not differ between both groups of defeated gilts and levels had returned within 30 min. Apparently, hypercortisolism is maintained in defeated isolates, despite recovery in ACTH levels. This may either present an enhancement in adrenal responsiveness to ACTH, regulatory changes in hypothalamic-pituitary hormones other than ACTH, or extrapituitary mechanisms (Levine et al., 1997). Not only hypercortisolism was prolonged in defeated isolates: this was also observed for changes in blood cellular immunity. While percentages of circulating leucocytes subsets were changed for at least 3 days in defeated isolates, values were 'normalized' within 1 day in defeated pair-housed animals. It may be suggested that a prolonged hypercortisolism maintains a redistribution in circulating leucocytes, but we were not able to detect differences in baseline (salivary) cortisol between the groups at 2 days after the defeat stress. When challenged in the repeated NET at 2 days after defeats, adrenocortical responsiveness of defeated isolates was similar to that in the test prior to defeats. However, in the test at day 2, the response was decreased in their pair-housed counterparts, as was also found in the control groups. A similar pattern was observed for locomotory behaviour in the latter NET, remaining at high levels in defeated isolates, but being reduced in the other groups.

We argue that the effect of the social environment shortly after defeat is related to a stress-reducing effect of an established and stable social relationship rather than being a consequence of additional stress caused by social isolation. The latter procedure had no (or little) consequences for gilts in the short term (isolated

controls). Restoring a stable social relationship, following a highly unstable social situation (during defeat), may offer defeated animals an improved ability to overcome the effects of the social stress. Such an effect of housing conditions has also been shown in a study with defeated rats (Ruis et al., 1999), in which adverse effects of acute social defeat were greatly reduced in animals which returned to stable social groups, compared to isolated animals. The amelioration of stress responses by the presence of members of the same species is called social support. In general, social support cannot be provided by any conspecific, but the ability to act as an 'arousal-reducing structure' is restricted to bonding partners (Sachser et al., 1998). In our study, social bonds existed between litter-mates, and it has been shown that this type of relationship is among the strongest in pigs (Petersen et al., 1989). Our results indicate that the presence of a familiar companion per se, irrespective of dominance relationships, may ameliorate responses to defeat stress. This may be due to high social stabilities, provided by established relationships between the members of a pair, resulting in predictable behaviour (Sachser et al., 1998). We finally hypothesize that, when benefitting from processes of social support, negative effects of mixing may be moderated by bringing subgroups of litter-mates together. This may reduce the number of defeats and may increase social buffering. As shown in an outdoor environment, familiar pigs tend to form subgroups, whereas unfamiliar pigs maintain separate rest areas for over 6 months (Stolba and Wood-Gush, 1984).

Conclusions

Our results emphasize the importance of established and stable social relationships between conspecifics. Instability, leading to social defeat, induces pronounced behavioural, endocrine and immunological changes. A stable social environment, on the other hand, not only reduces the occurrence of social stress, but group members may also buffer against the adverse effects of (social) stress (social support). Our data further emphasize that the pig is a socially living animal which requires (stable) social contact with conspecifics: social isolation leads to a higher vulnerability of gilts to subsequent environmental challenges.

Chapter 5

Long-term effects of social stress on anti-viral immunity in pigs

Physiology and Behavior, in press

J. de Groot[1,2], M.A.W. Ruis[1,2], J.W. Scholten[1], J.M. Koolhaas[2], W.J.A. Boersma[1]

[1] Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65,
8200 AB Lelystad, The Netherlands
[2] Department of Animal Physiology, University of Groningen, P.O. Box 14,
9750 AA Haren, The Netherlands

Abstract

Mixing of unfamiliar pigs is common practice in intensive pig husbandry. Since pigs maintain a dominance hierarchy, mixing often leads to vigorous fighting. Apart from the negative impact that fighting has on welfare, there is evidence that the social stress associated with fighting suppresses immune function. In the present experiment we investigated the impact of mixing on specific long-term immune responses and protection against challenge infection after vaccination with pseudorabies virus (PRV). Specific-Pathogen-Free (SPF) pigs were mixed pairwise with an unfamiliar same-gender conspecific, or left undisturbed with a same-gender littermate at 3 days after vaccination with PRV. Half of the pigs were females (gilts) and half were castrated males (barrows). Mixing increased agonistic behaviour to the same degree in gilts and barrows. Cortisol concentrations in saliva and catecholamine excretion in urine were increased in mixed pigs, and these effects were independent of dominance status and gender. Subsequently, the effects of mixing, gender, dominance status and interactions between these factors on immune response parameters were studied. The main result was that mixed barrows showed suppressed immune responses after vaccination and increased clinical symptoms after challenge infection compared to control barrows. Mixed gilts however did not differ from control gilts. It also appeared that mixed dominants were more seriously affected than mixed subordinates were. We conclude that in some pigs, social stress after mixing suppresses the immune response to a viral vaccine and consequently impairs protection against challenge infection.

Introduction

Pigs that are kept in intensive husbandry systems are exposed to various stressors in their lives. One potent stressor that pigs are confronted with is mixing with unfamiliar conspecifics. Most pigs are mixed immediately after weaning and sometimes also at a later stage to form growth cohorts that will be ready for slaughter simultaneously. Mixing of unfamiliar pigs induces fighting for dominance in the first days after mixing (Jensen, 1994; Meese and Ewbank, 1973). Both the physical activity associated with fighting, and the psychological stress associated with loosing the fight (in the subordinate animal) or threat to control (in the dominant animal) will cause physiological changes. Cortisol and catecholamine levels are increased after mixing of unfamiliar pigs, and dominance status (Fernandez et al., 1994; McGlone, 1990; Otten et al., 1999) and coping style

(Koolhaas and Bohus, 1989; Koolhaas et al., 1999) may influence the magnitude of various stress-hormone responses.

It is known that cortisol and other neuroendocrine components of the stress response affect the immune system (Griffin, 1989; Kelley, 1980, 1985). In pigs, an increase in cortisol concentration has been shown to suppress proliferation of lymphocytes to mitogens (Brown-Borg et al., 1993; Johnson et al., 1994; Wallgren et al., 1994), natural killer cell activity and neutrophilic chemotaxis (Salak Johnson et al., 1996, 1997). Thus, it might be expected that the immune system is negatively affected after mixing of unfamiliar pigs. Indeed, it has been shown that the swelling in response to intradermal injection of phytohaemagglutinine (PHA, a mitogen) was suppressed in pigs that were mixed immediately after injection of PHA (Ekkel et al., 1995a; Moore et al., 1994). Also, proliferation of lymphocytes in response to mitogens was affected after mixing, but this effect depended on the dominance status of the pig after mixing. Lymphocyte proliferation was increased in dominant pigs, but decreased in subordinate pigs when compared with pre-mixing levels (Tuchscherer et al., 1998). In contrast, it was also reported that mixing immediately or 2 weeks after weaning, did not suppress mitogen-induced lymphocyte proliferation at 1 day after mixing, nor did it suppress the swelling in response to PHA-injection and the antibody-response to sheep red blood cells (Blecha et al., 1985).

One of the factors leading to discrepancies between different studies of stress-effects on immunity may be that these studies tend to neglect the dynamic character of the immune system. The measurement of one immune parameter (mitogen-induced lymphocyte proliferation or response in the PHA-skin test) at one specific time-point after a stressor is not representative of the entire immune status of the animal. Thus the results of such studies are not easily interpretable towards stress-effects on immune responses (Dantzer, 1997; Dantzer and Kelley, 1989). It is known for example, that the anti-viral immune response is able to restore to control levels quickly after stress-induced suppression (De Groot et al., 1999). Also, the immune system is operated via many redundant pathways, and thereby compensation for the suppression of one specific immune parameter may occur. Thus, effects of stress on immunity should be measured on many time-points after the stressor, and several immune parameters should be taken into account, to be able to extrapolate the results towards the immune status of the animal. Based on these arguments, we designed an experiment to answer the following question: Does mixing of unfamiliar pigs affect the specific immune response against a viral vaccine and subsequent protection against challenge

infection with the pathogenic virus? And if so, does this effect depend upon gender and dominance status of the pig?

For this vaccination/challenge experiment, pseudorabies virus (PRV) was used. PRV is a herpes virus that causes Aujeszky's disease in pigs (Mettenleiter, 1994). The timing of the stressor relative to the moment of vaccination is known to influence the magnitude and direction of the effect of the stressor on the immune response. This has been shown in mice, where footshocks only suppressed the immune response against sheep red blood cells when given at 72 hours following immunization. No such effect was seen when footshocks were given at 0, 24, 48 or 95 hours after immunization (Zalcman et al., 1988). We have observed in mice that immunosuppression occurred when a social stressor was given at 3 or 6 days after immunization, but not when given at 1 day after immunization with PRV (De Groot et al., 1999, unpublished observations). Thus in the present study pigs were mixed at 3 days after vaccination. The specific immune response against PRV was measured weekly after vaccination to be able to compare the development of the immune response in mixed and control pigs. Finally, pigs were challenged with the pathogenic wild-type virus at 42 days after vaccination, and the memory immune response against PRV as well as symptoms of clinical illness were evaluated.

Materials and Methods

Animals and housing

Outbred Dutch Landrace pigs used in this experiment were obtained from the Specific-Pathogen-Free (SPF) herd of the Institute for Animal Science and Health (ID-Lelystad) in Lelystad, The Netherlands. The pigs were born from three multiparous sows (which were all impregnated with the same boar) and were free of antibodies against pseudorabies virus (PRV) before the start of the experiments. Tattooing, weighing, teeth clipping and tail docking of piglets was carried out at 1 or 2 days of age. All piglets received an iron-injection at 1 or 2 days of age. Male piglets were castrated without anaesthesia at 3 weeks of age, and all piglets were weaned at 4 weeks of age. Litter sizes were 9, 11, and 12 piglets for the 3 sows, and mean(±SEM) birthweights were respectively 2007±73, 1436±49, and 1612±83 g for these litters. Four gilts and four barrows per sow were randomly selected for use in the experiment and transported to the experimental SPF-unit of the institute at the day of weaning.

Piglets were then housed in pairs consisting of either two gilts or two barrows from one litter, in pens (1 x 2 m) with a solid concrete floor covered with sawdust. Three such pens were located in one experimental unit and 4 similar units

were used in the entire experiment. Pens were cleaned daily and throughout the experiment water and food were available ad libitum. Environmental temperature was kept at 20^0C. Artificial lights were on from 06.00-18.00 h with no daylight visible in the rooms. Between 05.00 and 06.00 h and between 18.00 and 19.00 h dim lights were on.

Experimental design

A schematic overview of experimental manipulations is presented in Figure 1. The selected piglets from each sow were housed in pairs of two gilts or barrows (from one litter) after weaning. Five days prior to mixing procedures, animals in each pair were subjected to a food-competition test. This was done only for design of pairs that were to be mixed, that is, to minimize variation amoung new pairs of pigs after mixing, new pairs were designed to consist of a 'winner' and 'loser' of the food-competition test.

Overview of experimental procedures

we	weaning
p	pair-housing
b	blood samples for immune response parameters (proliferation, antibodies, cytokines)
f	food-competition test
v	vaccination with an attenuated live strain of PRV
m	mixing
r	acute response to mixing (behaviour, cortisol, catecholamines)
c	challenge infection with the wild-type strain Northern Ireland Aujeszky 3 (NIA_3) of PRV
d	daily clinical symptoms (body temperature, body weight, virus excretion)

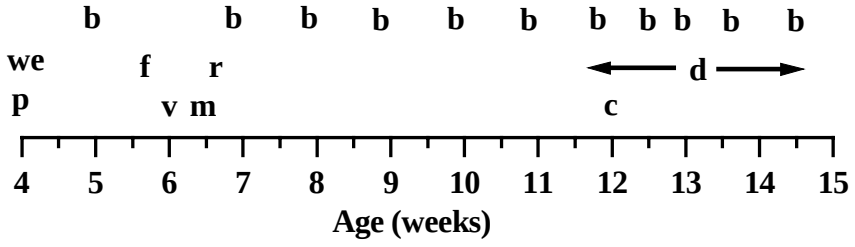

Figure 1. A schematic presentation of experimental manipulations and of parameters measured during the experiment.

The two pigs in each pen shared one food hopper and were not able to eat simultaneously. After 12 hours of food deprivation, food was added to the food hopper, and subsequent agonistic behaviour was videotaped during 15 min. The results of the food competition test were not quantitated, but rather a qualitative judgement was given after observing the videotapes. In this judgement, the pig which replaced the other pig more often at the food hopper, was considered to be the 'winner'. Note that this test was only performed to minimize variation between mixed pairs, and that dominance as a factor in the statistical analysis was always the dominance in the pairs after mixing, and in control pairs in the same period, based on behavioural observations during a period of 3 days following mixing.

At 6 weeks of age all piglets were vaccinated intramuscularly (in the neck) with 1.10^6 plaque forming units (PFU) of the attenuated strain 783 of PRV (Moormann et al., 1990). No adjuvant was used in the vaccine. Vaccination of pigs with an attenuated live strain of PRV protects pigs against infection with the wildtype PRV (Van Oirschot et al., 1991). Three days after vaccination, half of the piglets were mixed with a same-gender conspecific from another litter. The new pen was unfamiliar to both piglets and from this time onwards the piglets stayed in this pen, thus being forced to fight for dominance. The control piglets were left undisturbed in their home pens. For practical reasons, control gilts, control barrows, mixed gilts and mixed barrows were housed in separate units. The 3 different litters were balanced over treatments, thus from 1 sow, 2 gilts were designed to the control group, 2 gilts were designed to the mixing group, 2 barrows were designed to the control group and 2 barrows were designed to the mixing group. All pigs were challenged intranasally with 5.10^4 PFU of the wild-type strain Northern Ireland Aujeszky 3 (NIA_3) of PRV in each nostril at 42 days after vaccination. Stocks of the vaccine and wild-type virus strain were prepared and stored as described previously (Kimman et al., 1995).

The response to mixing was evaluated in three ways: (1) behaviour was recorded on video for 3 days after mixing; (2) cortisol concentration was measured in saliva samples taken 5 min before, and 1, 2, 4, 24 and 48 hours after mixing; and (3) adrenaline and noradrenaline concentration were measured in urine samples collected in the early morning after mixing.

Immune responses to vaccination and challenge were measured in blood. Pigs were restrained and blood was collected from the superior vena cava at days -8, 6, 13, 20, 27, 34, 41, 45, 48, 52 and 59, relative to vaccination (day 0) and challenge (day 42). Blood was allowed to clot, centrifuged and serum was stored at -20^0C for later analysis of anti-viral antibody-titers. Heparinized blood was used

for lymphocyte purification and subsequent in vitro culture to determine virus-specific lymphocyte proliferation and cytokine production (the latter assay was only performed at days 6, 20, 48 and 59 after vaccination).

Clinical scores of illness were determined by daily measurement of body temperature (rectal) and body weight from day 41 until day 59 (1 day before challenge until 17 days after challenge). Virus excretion was determined in swabs of oropharyngeal fluid (OPF) that were collected daily from restrained pigs in the same period.

Behavioural analysis

The behaviour of both mixed and control pigs during the first 3 days after mixing was analyzed with the Observer® software program (Noldus Information Technology, Wageningen, The Netherlands). The frequency of the following behaviours aimed at the penmate, were scored per pig: bites (directed to all parts of the body), headknocks (rapid thrusting the head or snout upwards or sidewards to any part of the body), threats (aggressive interactions not involving physical contact), and pushes (replacing the other animal). The first 2 hours after mixing were recorded by continuous sampling, the next 72 hours by time-lapse recording (72 hours on a 180 min tape). Behaviour was only analyzed during the light period between 06.00 and 18.00 h. Agonistic behaviours are expressed as actions per pair during the first 2 hours after mixing (~10.00-12.00 h), the rest of day 1 (~12.00-18.00 h) , day 2 (06.00-18.00 h), and day 3 (06.00-18.00 h) of mixing. The pig that displayed the majority of the offensive agonistic actions in the pair was identified as the dominant, and the other pig in the pair was the subordinate (McGlone, 1985; Rushen and Pajor, 1987).

Hormones

Salivary cortisol. Saliva was collected by allowing the pigs to chew on two large cotton-buds, until they were thoroughly moistened. Samples were stored at -20^0C until analysis for cortisol concentration with a solid-phase radioimmunoassay kit (Coat-a-Count Cortisol® TKCO, Diagnostic Products Corporation, Apeldoorn, the Netherlands) modified for pig cortisol (Ruis et al., 1997). Cortisol in saliva is essentially in the free, biologically active form, and is a good indication of cortisol concentration in blood plasma (Kirschbaum and Hellhammer, 1989; Parrott et al., 1989).

Urinary catecholamines. Urine samples were collected with small buckets on a stick, between 05.00 and 07.00 h in the morning after mixing. For practical

reasons, urine samples from control animals were collected one day later. Samples were stored on ice until processing. Urinary catecholamines (noradrenaline and adrenaline) were assayed using a high performance liquid chromatography (HPLC) procedure with electrochemical detection (Ruis et al., 2001), following a two step extraction. One hundred µl urine was extracted using the sephadex column extraction as described previously (Westerink and Koolstra, 1986). This first clean-up step resulted in a 2.5 ml extract of the urine sample. One ml of this extract was taken and subjected to the liquid extraction (twice) as described previously (Smedes et al., 1982). One hundred µl of the extract obtained after this second clean-up step was injected into the HPLC system. Detection limits were 35 pg/ml for noradrenaline and 55 pg/ml for adrenaline. Creatinine levels were determined using a colorimetric quantitative reaction (Boehringer PAP-method). Color intensity was measured at 510 nm. Intra- and inter-assay CV were 2 and 5%, respectively. To correct for variable dilutions of urine related to water intake, catecholamine levels will be presented as proportions of creatinine: noradrenaline/creatinine and adrenaline/creatinine ratios.

Immune response parameters

Anti-viral antibodies. Anti-viral IgM, IgG1, and IgG2 levels were determined in serum samples with ELISA as described previously (Kimman et al., 1992). Titers are expressed as optical density at a serum dilution of 320x (IgM) or $\log_{10}$ of the slope of the fitted line in an optical density versus inverted dilution plot (IgG1 and IgG2).

Anti-viral lymphocyte proliferation. Peripheral blood mononuclear cells were enriched for lymphocytes by centrifugation on a Ficoll®-paque discontinuous 1-step density gradient (Pharmacia Biotech, Uppsala, Sweden). After washing, lymphocytes were resuspended to a concentration of 5.10^6 lymphocytes/ml in Dulbecco's Modified Eagle Medium (DMEM-alpha), supplemented with 10% pig serum (obtained from SPF-pigs from the herd of the institute and filtrated through a 0.45 µm filter), 5% antibiotic mix (containing penicillin, streptomycin, amphoterrin, polymycin and kanamycin), 2 mM glutamin, and 5.10^{-5} M 2-mercaptoethanol. Lymphocytes were cultured in triplicate: 100 µl of cell suspension per well was cultured in flatbottom 96 well plates (Costar®, Corning Inc., New York, USA), supplemented with 100 µl complete medium or 100 µl of pseudorabies virus strain NIA_3 in complete medium (multiplicity of infection: 1). Cultures were pulsed with 0.4 µCi per well methyl-^{3}H-thymidine® (Amersham, The Netherlands) for the last 4 hours of culture, immediately after isolation of the

cells (ex-vivo) and after 4 and 6 days of culture. Cells were then harvested on glassfiber filters with an automatic cell harvester (Tomtec®, EGG, Nieuwegein, The Netherlands). Incorporation of radioactivity was measured after addition of scintillation fluid, with a β-counter (Wallac®, EGG, Nieuwegein, The Netherlands) and expressed as mean corrected counts per minute (ccpm) of triplicates.

Cytokine production. Cell-free supernatant from lymphocytes that were cultured with or without PRV restimulation was collected at 4 days after start of culture. At this time-point, IL-10 and IFN-gamma concentrations in supernatant were found to be optimal. Lymphocytes were cultured as described for the proliferation assay and supernatant was stored at -70^0C until assaying. Serial dilutions of supernatant were analyzed for IL-10 and IFN-gamma concentration using commercial ELISA-kits® (Biosource, CA, USA). A standard of recombinant cytokine was included in each plate, and a linear fit of $\log_{10}$ optical density and $\log_{10}$ concentration of recombinant cytokine was used to calculate the concentration of cytokine in the supernatant samples. Intra- and inter-assay CVs were 4.2 and 7.8%, respectively, for IFN-gamma, and 5.1 and 7.2%, respectively, for IL-10.

Clinical scores of illness

Body temperature. Rectal temperature was measured with a digital thermometer. Pigs were allowed to walk freely after the thermometer was inserted, and a beep would signal the experimenter that a stable temperature had been reached.

Virus excretion. Oropharyngeal fluid (OPF) was collected on sterile gauzes that were swabbed in the throat of the animal under restraint. The concentration of excreted virus in OPF was determined by titration on a swine kidney cell-line (SK6), as described previously (Van Rooij et al., 2000).

Statistics

For parameters that were measured at many time-points (lymphocyte proliferation, IgG1 and IgG2a antibody levels, virus excretion, delta body weight after challenge, body temperature after challenge), statistical analysis was performed on the area under the curve (AUC) of the response. The AUC was calculated by addition of the exact areas between each two successive time-points.

Cortisol levels, and immunological and clinical illness data were analyzed with analysis of variance (ANOVA) for main effects of treatment (mixing or control), gender (barrow or gilt) and dominance (dominant or subordinate, as

established after mixing; for the control groups as determined in the same period), and for the interaction between these factors. If an interaction effect was found (p<0.10), significance of differences between specific groups was calculated using the least significant differences (lsd's) from the combined analysis. When there were no interaction effects (p>0.10), they were omitted from the model. Virus-excretion was not only analyzed as AUC, but also per time-point, because differences in kinetics of virus excretion between groups were expected. Virus excretion data were analyzed for main effects of treatment (mixing or control), gender (barrow or gilt), and dominance (dominant or subordinate) and the interaction between these factors with a general linearized model (GLM). A poisson distribution and log-transformation were incorporated in the GLM and the dispersion was estimated from Pearson-chi. When no significant interaction effects were found (p>0.10), they were omitted from the model. For catecholamines in urine the data set was incomplete, and a Restricted Estimated Maximum Likelihood (REML) procedure was followed.

The above mentioned parameters were analyzed on the animal level, assuming that pigs in one pair were independent with respect to these data. However, behavioural data were analyzed on the pair level, assuming that the amount of aggression displayed by each animal was dependent on the other pig in the pair. Behavioural data were analyzed for main effects of treatment (mixing or control), gender (barrow or gilt), and the interaction between gender and treatment with a GLM. A poisson distribution and log-transformation were incorporated in the GLM and the dispersion was estimated from Pearson-chi. When no significant interaction between treatment and gender was found (p>0.10) the interaction was omitted from the model and behavioural data were analyzed for main effects of treatment and gender only.

Because the costs of experimenting with SPF-pigs and pathogenic material did not allow us to test large groups of pigs, a number of variables did not reach significance at the 0.05 level. However, we feel that tendencies for effects as they were found in the present study are of interest for the generation of ideas on possible mechanisms of stress effects on immunity in pigs. Therefore, not only significancies at the 0.05 level, but also tendencies at the 0.10 level will be reported. All statistical calculations were done in Genstat 5® (1993). Unless stated otherwise, data are presented as mean±SEM.

Results

Response to mixing

Behaviour. Mixed pigs displayed significantly more agonistic actions than control pigs during the first 3 days after mixing (Table 1). Agonistic actions were most frequent during the first 2 hours after mixing but remained elevated at days 2 and 3 of mixing (Table 1). Gender did not affect the frequency of the agonistic actions (p>0.10 for all periods).

Table 1. Agonistic behaviour in control and mixed pairs.

		Frequencies of agonistic actions				
		Control pairs		Mixed pairs		
		Mean±SEM[1]	% by dominant[2]	Mean±SEM[1]	% by dominant[2]	p[3]
First 2 hours	Bites	5.8±3.1	85.7	132.2±39.6	73.5	0.11
	Headknocks	3.8±1.9	73.9	20.2±5.0	59.5	0.04
	Threats	6.0±3.9	91.7	61±13.3	87.7	0.04
	Pushes	6.8±2.6	63.4	89±30.9	70.0	0.10
Rest of day1	Bites	3.7±2.0	90.9	30.2±4.4	68.5	0.02
	Headknocks	3.3±1.5	60.0	11.7±1.9	72.9	0.02
	Threats	4.3±1.8	100	23.3±7.1	96.4	0.06
	Pushes	16.0±5.1	65.6	42.7±3.8	66.8	0.01
Day 2	Bites	3.2±2.2	89.5	24.2±6.5	77.9	0.05
	Headknocks	2.0±1.1	83.3	12.5±2.3	78.7	0.02
	Threats	1.3±0.5	100	22.8±6.1	96.4	0.07
	Pushes	16.5±5.0	62.6	48±7.6	59.0	0.02
Day 3	Bites	0.8±0.4	60.0	14.3±4.0	75.6	0.08
	Headknocks	2.5±1.4	93.3	15.2±4.0	74.7	0.04
	Threats	1.5±0.8	100	14.2±2.2	94.1	0.02
	Pushes	15.5±3.7	61.3	40±5.7	57.9	0.02

[1]Mean(±SEM) of numbers of agonistic interactions within pairs of pigs (6 control and 6 mixed pairs). [2]Mean percentage of interactions performed by the pigs that were assigned the dominant status. [3]Significance of the effect of mixing on agonistic interactions, based on comparisons of pair-means.

In both mixed and control pairs the dominant pig was identified as the pig displaying the majority of the agonistic actions. The difference between the dominant and the subordinate pig was most clearly seen in the display of threats (Table 1). This behaviour was defined as one pig threatening to show offensive behaviour, to which the penmate responded by showing defensive behaviour. In mixed pairs, there was a sow-effect on dominance. Piglets, both barrows and gilts, from one sow always became dominant over piglets of another sow. Also, in the mixed pairs the dominant pig was always the heaviest pig of the pair, whereas in the control pairs the dominant pig was the heaviest in 3 out of 6 pairs (data not shown).

Cortisol. Cortisol concentration in saliva increased after mixing (Table 2). Mixed pigs showed higher cortisol levels than control pigs at 1 hour after mixing. No effects of gender or dominance on this initial rise in cortisol levels were found ($p>0.10$). However, cortisol levels at 24 hours after mixing were significantly affected by gender (gilts: 1.19±0.09 ng/ml; barrows: 1.65±0.14 ng/ml; $p<0.05$).

Table 2. Salivary cortisol concentrations (mean±SEM) after mixing.

	Salivary cortisol (ng/ml)		
Hours after mixing	Control pigs	Mixed pigs	p^1
0	1.57±0.16	1.24±0.12	0.11
1	1.40±0.15	2.71±0.53	0.02
2	1.27±0.12	2.08±0.43	0.09
4	1.63±0.17	1.23±0.13	0.08
24	1.49±0.12	1.30±0.15	0.47
48	1.19±0.16	1.50±0.22	0.26

[1]Significance of differences between control pigs (n = 12) and mixed pigs (n = 12).

Catecholamines. Levels of adrenaline and noradrenaline were determined in urine collected in the morning of the day after mixing. Because of the difficulty of being at the right moment and in the proper position to be able to 'catch' the urine, these data sets are incomplete. Data are available for 3 mixed gilts, 5 control gilts, 4 mixed barrows and 3 control barrows. Statistical analysis was therefore limited to main effects of treatment, gender and dominance. It appeared that adrenaline excretion (expressed as a proportion of creatinine: A/C x 10^{-6}) was significantly affected by treatment (controls: 0.70±0.13; mixed: 1.68±0.37;

$p<0.05$), but not by gender and dominance. Noradrenaline excretion (expressed as a proportion of creatinine: N/C x 10^{-6}) was affected by both treatment and dominance. Mixed pigs showed higher noradrenaline excretion than control pigs (controls: 11.20±1.00; mixed: 18.19±2.50; $p<0.05$), and dominant pigs tended to excrete more noradrenaline than subordinate pigs (dominants: 17.31±2.58; subordinates: 11.97±1.42; $p=0.07$).

Immune response parameters

Lymphocyte proliferation. Proliferative responses were measured in two different ways. In the first assay the proliferation immediately after isolation of peripheral blood mononuclear cells was measured in the absence of virus in vitro. This is called the ex-vivo proliferation, since it reflects the proliferation that is due to in vivo activation of the cells. In the second assay the proliferation in response to in vitro viral restimulation was measured.

Typically, there is an increase in ex-vivo proliferation levels between the sample before vaccination and the sample at 6 days after vaccination. A significant interaction between treatment and dominance on the increase in ex-vivo proliferation was seen ($p<0.05$). Comparison between groups showed that ex-vivo proliferation increased significantly more in control dominants than in mixed dominants (control dominants: 7570±1723.4 ccpm; mixed dominants: 1204±1389.0 ccpm; $p<0.05$), whereas there was no difference between control and mixed subordinates (control subordinates: 3732±691.9 ccpm; mixed subordinates: 3862±1558.2 ccpm; $p>0.10$).

Ex-vivo proliferation also increased between the sample before and 6 days after challenge infection. No effects of treatment, dominance or gender on this increase were found ($p>0.10$; data not shown).

Proliferation in response to in vitro viral restimulation reflects the presence of immunological memory. This PRV-induced proliferation was detectable from 6 days after vaccination onward and reached a plateau level at 13 days after vaccination (Figure 2). The response to vaccination was expressed as the area under the curve (AUC) from 8 days before vaccination until 41 days after vaccination. There was a tendency for an interaction effect between gender and treatment on the AUC of the PRV-induced proliferation ($p=0.07$). In a comparison between groups, it appeared that mixed barrows showed a significantly lower response than control barrows (controls: 1.77±0.261 x 10^6; mixed: 1.11±0.158 x 10^6; $p<0.05$) (Figure 2B). However, mixed and control gilts showed a similar response (controls: 1.44±0.332 x 10^6; mixed: 1.63±0.171 x 10^6; $p>0.10$) (Figure

2A). The AUC of PRV-induced proliferation from 8 days before until 41 days after vaccination was also significantly affected by dominance. Dominant pigs showed a higher response than subordinate pigs (dominants: 1.74±0.159 x 10^6; subordinates: 1.23±0.164 x 10^6; p<0.05) (Figure 3).

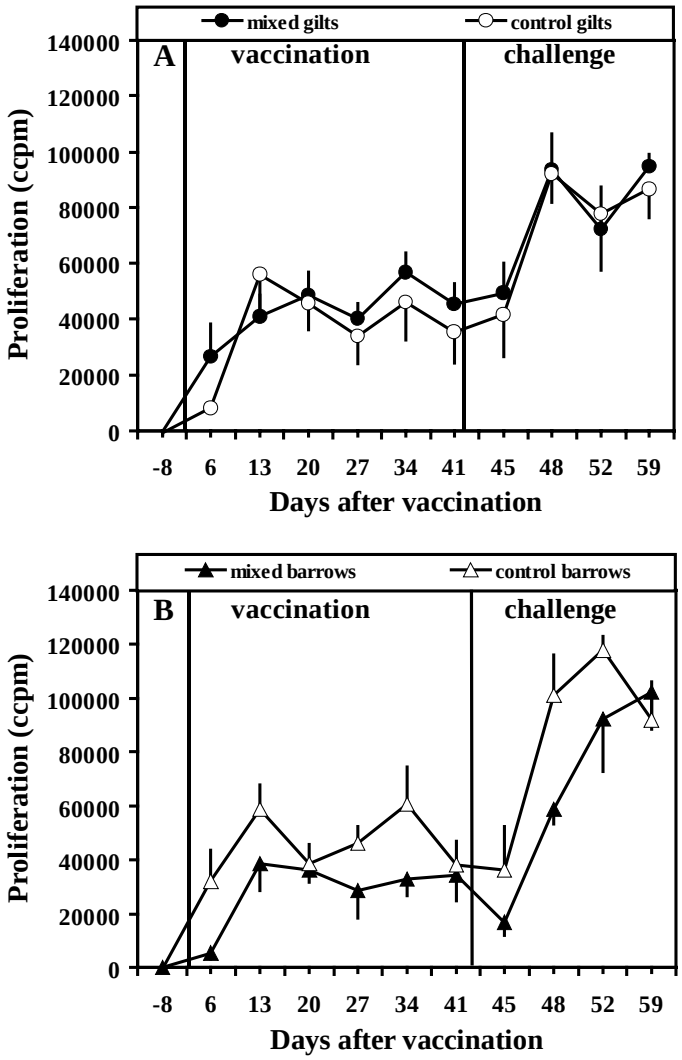

Figure 2. Lymphocyte proliferation (mean±SEM) in response to in vitro restimulation with pseudorabies virus (PRV), after vaccination (day 0) and challenge (day 42). (A) control gilts (n = 6) and mixed gilts (n = 6); and (B) control barrows (n = 6) and mixed barrows (n = 6).

After challenge, the in vitro restimulated proliferation increased 2-3 times, reflecting expansion of memory lymphocytes in vivo (Figures 2 and 3). There were

no significant effects of gender, treatment and dominance on the AUC from 41 days until 59 days after vaccination.

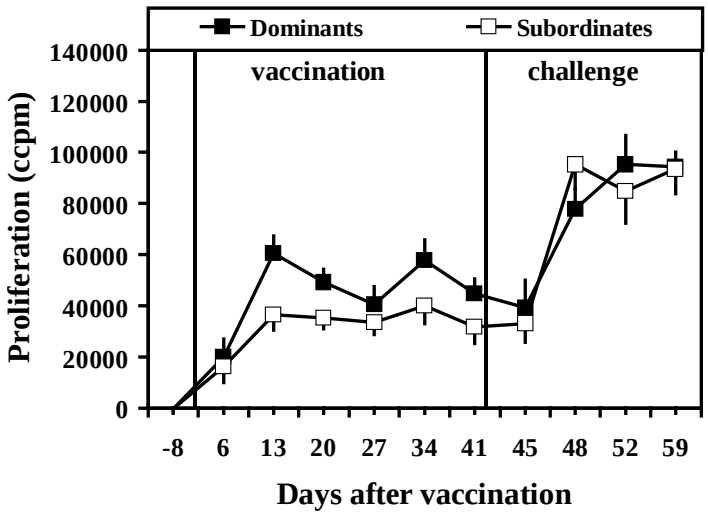

Figure 3. Lymphocyte proliferation (mean±SEM) in response to in vitro restimulation with pseudorabies virus (PRV), after vaccination (day 0) and challenge (day 42), in dominant pigs (n = 12) and subordinate pigs (n = 12).

Antibody responses. Anti-viral IgM antibodies were detectable on days 6, 13 and 20 after vaccination. There was a tendency for an interaction between gender and treatment on the AUC from days 6-20 after vaccination (p=0.08). Comparison of groups showed that mixed barrows had a significantly lower IgM response than mixed gilts (p<0.05), but that the difference between mixed and control barrows as well as the difference between mixed and control gilts was not significant (p>0.10; control barrows: 7.34±1.19; control gilts: 7.15±0.84; mixed barrows: 4.91±0.47; mixed gilts: 8.71±1.47).

IgG1-antibodies to PRV were detectable from day 13 after vaccination onward and reached a plateau level between days 20 and 27 (Figure 4A). Gender, treatment or dominance did not significantly affect the AUC between 8 days before vaccination and 41 days after vaccination. IgG1-antibody titers rose in response to challenge (Figure 4A). The AUC for IgG1 from 41 days until 59 days after vaccination (this is the actual response to challenge since the AUC was corrected for the titer on the day before challenge) was significantly affected by gender. Gilts showed a lower IgG1 response to challenge than barrows (gilts: 9.40±1.50; barrows: 13.71±1.29; p=0.05).

Anti-PRV IgG2 antibodies showed approximately the same kinetics as IgG1 antibodies (Figure 4B). For the AUC of the IgG2 response to vaccination (day 8 before vaccination until day 41 after vaccination), a significant effect of gender was seen. Gilts showed a higher IgG2-response to vaccination than barrows (gilts: 51.2±2.41; barrows: 43.9±2.12; $p<0.05$). No significant effects of treatment or dominance were seen ($p>0.10$). After challenge, IgG2 titers increased (Figure 4B). A tendency for an interaction effect between gender and treatment on the AUC for IgG2 from 41 days until 59 days after vaccination (corrected for the titer on the day before challenge: thus the actual response to challenge) was found ($p=0.09$).

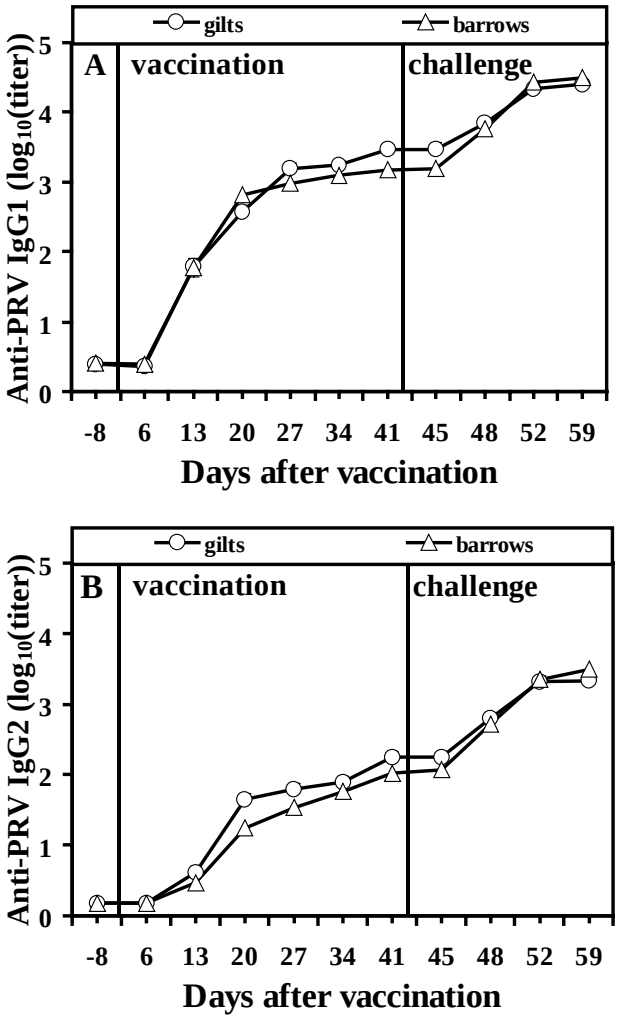

Figure 4. Anti-PRV antibody titers (mean±SEM) after vaccination (day 0) and challenge (day 42) in gilts (n = 12) and barrows (n = 12). (A) IgG1; and (B) IgG2.

Comparison of groups showed that mixed barrows had a stronger IgG2 response to challenge than mixed gilts ($p<0.05$). However the difference between mixed and control barrows as well as between mixed and control gilts was not significant ($p>0.10$; control barrows: 13.0±1.80; control gilts: 13.0±2.50; mixed barrows: 17.2±1.38; mixed gilts: 10.0±1.70).

The balance between IgG1 and IgG2 antibody responses was also evaluated. After vaccination the IgG1/IgG2-ratio was significantly affected by gender. Gilts showed a lower IgG1/IgG2-ratio than barrows (gilts: 1.82±0.07; barrows: 2.09±0.10; $p<0.05$). No effects of treatment or dominance were found. After challenge the IgG1/IgG2-ratio tended to be lower in gilts than in barrows (gilts: 0.75±0.07; barrows: 0.91±0.05; $p=0.07$). Also there was a tendency for an effect of dominance on the IgG1/IgG2-ratio after challenge (dominants: 0.91±0.04; subordinates: 0.76±0.08; $p=0.08$).

Cytokine production. IFN-gamma production in response to viral restimulation in vitro was measured at 6 and 20 days after vaccination and at 6 and 17 days after challenge. Table 3 shows that virus-specific production of IFN-gamma was already detectable at 6 days after vaccination, but was further increased between 6 and 20 days after vaccination. Challenge infection induced a 3-fold increase of IFN-gamma production. There was no background production of IFN-gamma in cultures that were not restimulated with virus.

Table 3. In vitro production of IFN-gamma and IL-10 (mean±SEM) in response to restimulation of lymphocytes with pseudorabies virus (PRV).

	IFN-gamma (pg/ml)	IL-10 (pg/ml)[1]
6 days after vaccination	2032±373	33±8.3
20 days after vaccination	3415±387	104±18.1
6 days after challenge	9632±746	238±31.0
17 days after challenge	7690±917	165±22.9

[1]Difference between IL-10 production in restimulated cultures and non-restimulated cultures.

At 6 days after vaccination there was a tendency for an interaction effect between gender and treatment on IFN-gamma production ($p=0.07$). Comparison of groups showed that the IFN-gamma production tended to be lower in mixed than in control barrows (control: 2190±443 pg/ml; mixed: 962±254 pg/ml; $p<0.10$), whereas there was no significant difference between mixed and control gilts

(control: 1932±519 pg/ml; mixed: 3044±1259 pg/ml; $p>0.10$). At 20 days after vaccination there was a tendency for a dominance effect on IFN-gamma production (dominants: 4099±582 pg/ml; subordinates: 2732±424) pg/ml; $p=0.08$). At 6 days after challenge there was a tendency for an interaction between gender and dominance on IFN-gamma production ($p=0.09$). When groups were compared it appeared that dominant gilts tended to have a higher IFN-gamma production than subordinate gilts ($p<0.10$), and that dominant gilts had a significantly higher IFN-gamma production than both dominant and subordinate barrows ($p<0.05$) (dominant barrows: 7572±1140 pg/ml; dominant gilts: 12943±1806 pg/ml; subordinate barrows: 8687±1074 pg/ml; subordinate gilts: 9326±997 pg/ml). At 17 days after challenge there was a tendency for a higher IFN-gamma response in gilts than in barrows (gilts: 9351±1591; barrows: 6028±693 pg/ml; $p=0.07$).

Production of IL-10 was also measured at 6 and 20 days after vaccination and at 6 and 17 days after challenge. There was a background production of IL-10 in cultures that were not restimulated in vitro, and thus the difference between restimulated and not restimulated cultures was taken as measure for virus-specific IL-10 production. Table 3 shows that the kinetics of IL-10 production after vaccination and challenge resemble the kinetics of IFN-gamma production. However, production of IL-10 was much lower than production of IFN-gamma. At 6 days after vaccination virus-specific IL-10 production was not evident in all pigs, as some pigs showed similar production levels with and without viral restimulation. It appeared that there was a significant interaction between gender and treatment for virus-specific IL-10 production at 6 days after vaccination ($p<0.01$). IL-10 levels were lower in mixed barrows than in control barrows (control: 65.0±5.6 pg/ml; mixed: 4.9±4.9 pg/ml; $p<0.05$), whereas the difference between mixed gilts and control gilts was not significant (control: 33.7±18.7 pg/ml; mixed: 43.2±10.7 pg/ml; $p>0.10$). At 20 days after vaccination there were no significant effects of treatment, gender or dominance on virus-specific IL-10 production ($p>0.10$). Production of IL-10 at 6 days after challenge was significantly affected by gender. Barrows showed higher virus-specific IL-10 production than gilts (gilts: 178±38.0 pg/ml; barrows: 299±43.5 pg/ml; $p<0.05$). At 17 days after challenge there were no effects of treatment, gender or dominance on virus-specific IL-10 production ($p>0.10$).

The balance between production of IFN-gamma and IL-10 may be regarded as an important indicator of the Th1/Th2-balance of the immune response. Thus, the effects of treatment, gender and dominance on the IFN-gamma/IL-10 ratio were analyzed. Since after vaccination IL-10 levels were low in some

animals, resulting in a very high IFN-gamma/IL-10 ratio, statistical analysis was limited to the data after challenge where both cytokines were produced in sufficient amounts in all pigs. Because the data did not show a normal distribution, they were log-transformed before analysis. For the IFN-gamma/IL-10 ratio at 6 days after challenge a significant interaction between treatment and dominance was found ($p<0.05$). Comparing groups it appeared that for the mixed pigs, dominant pigs showed a lower IFN-gamma/IL-10 ratio than subordinate pigs (mixed dominants: 1.51±0.14 (^{10}log (IFN-gamma/IL-10)); mixed subordinates: 1.79±0.09) (^{10}log (IFN-gamma/IL-10)); $p<0.05$). However for the control pigs, there was no significant difference between dominant and subordinate pigs (dominant controls: 1.70±0.16 (^{10}log (IFN-gamma/IL-10)); subordinate controls: 1.55±0.06 (^{10}log (IFN-gamma/IL-10)); $p>0.10$). The IFN-gamma/IL-10 ratio at 6 days after challenge was also significantly affected by gender. Gilts showed a higher ratio than barrows (gilts: 1.81±0.06 (^{10}log (IFN-gamma/IL-10)); barrows: 1.47±0.08 (^{10}log (IFN-gamma/IL-10)); $p<0.01$).

At 17 days after challenge there was a tendency for gilts to have a higher IFN-gamma/IL-10 ratio than barrows (gilts: 1.82±0.07 (^{10}log (IFN-gamma/IL-10)); barrows: 1.63±0.06 (^{10}log (IFN-gamma/IL-10)); $p=0.07$).

Clinical illness after challenge

Body weight. Increase in body weight stagnated or reversed after challenge (data not shown). The AUC for delta body weight was calculated between 1 day before challenge and 7 days after challenge since clinical symptoms disappeared in all pigs by that time. There were no significant effects of treatment, gender or dominance on the AUC for delta body weight.

Body temperature. A fever response was seen between 1 and 7 days after challenge (Figure 5). There was a tendency for an interaction effect between treatment and gender on the AUC for body temperature from 1 day before challenge until 7 days after challenge ($p=0.06$). It appeared that mixed barrows had a higher AUC for body temperature than mixed gilts ($p<0.05$). However the difference between mixed and control barrows as well as the difference between mixed and control gilts was not significant ($p>0.10$; control gilts: 11.23±0.59; mixed gilts: 10.17±0.48; control barrows: 11.93±0.75; mixed barrows: 13.23±0.54).

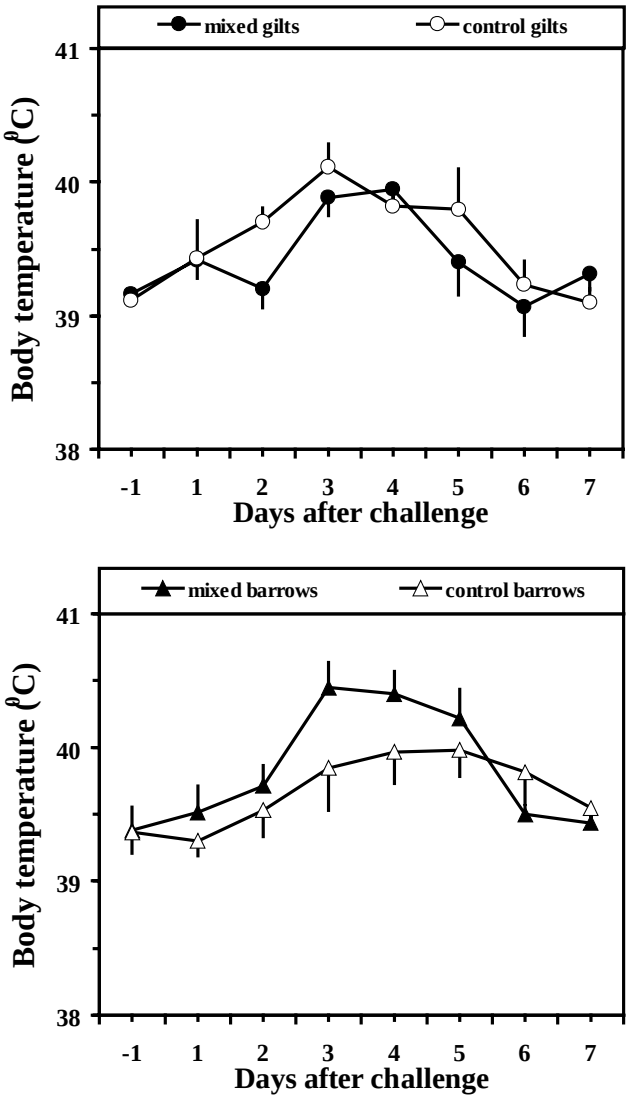

Figure 5. Body temperature (mean±SEM) after challenge with pseudorabies virus (PRV) in control gilts (n = 6) and mixed gilts (n = 6) (upper panel) and control barrows (n = 6) and mixed barrows (n = 6) (lower panel).

Virus excretion. Virus excretion was detected between 1 and 7 days after challenge. Virus excretion was analyzed as the AUC of ^{10}log(virus PFU/ml oropharyngeal fluid) in this period. It appeared that gilts excreted less virus than barrows (gilts: 15.62±1.98; barrows: 20.42±0.67; $p<0.05$). Also a tendency for an interaction between treatment and dominance was found ($p=0.07$). Mixed subordinates excreted significantly less virus than mixed dominants ($p<0.05$), and tended to excrete less virus then control subordinates ($p<0.10$). No difference

between control subordinates and control dominants was found (p>0.10; mixed dominants: 20.08±0.59; mixed subordinates: 13.94±2.09; control dominants: 18.30±2.07; control subordinates: 19.75±0.69).

Virus excretion per time-point was analyzed, because there might be differences in the kinetics of virus excretion between groups. No significant effects of treatment, gender, or dominance were found for any of the time-points (p>0.10) (data not shown).

Discussion

The present study shows that mixing of unfamiliar pigs can have a long-term modulating effect on the immune response against a viral vaccine. This effect is dependent on gender, with a long term suppression of the primary immune response and a higher fever-response after challenge found in mixed barrows, but not in mixed gilts. The effect of mixing is also dependent on dominance status, with mixed dominants showing a Th1/Th2-cytokine profile that was skewed towards Th2, and a higher virus excretion after challenge compared to mixed subordinates.

A different effect of mixing in barrows and gilts was found for many immune parameters. After vaccination, restimulated lymphocyte proliferation, IgM, IFN-gamma and IL-10 responses were lower in mixed barrows than in control barrows, whereas these responses were similar in mixed and control gilts. Thus, the primary immune response to vaccination was suppressed in mixed barrows, but not in mixed gilts. After challenge, it appeared that the secondary antibody response (IgG2) was higher in mixed barrows than in mixed gilts. It is known that after infection with PRV, a higher secondary antibody response is an indication of decreased protection against challenge infection (Kimman et al., 1995). In less protected pigs the lymphocyte proliferation response after challenge infection will be lower, and a relatively high antibody response can be seen. Finally, mixed barrows showed a stronger elevation of body temperature than mixed gilts after challenge infection. Thus the effect of mixing on barrows extended to protection against clinical symptoms of PRV-infection.

These results imply that barrows suffered from immunosuppression after mixing, whereas gilts did not. Other studies that have investigated effects of mixing on immune parameters in pigs, have not reported differences between barrows and gilts (Ekkel et al., 1995a; Hessing et al., 1995; Moore et al., 1994; Tuchscherer et al., 1998). However, in these studies larger groups per pen were used and gilts and barrows were combined in one group, which may explain the discrepancy with the

present study. Also, in contrast to the above mentioned studies, SPF-pigs were used in the present study. This allowed us to control for immunological disturbances by subclinical infections or obligatory vaccinations. Finally, as we argued in the introduction, the suppression of one immune parameter at one specific time-point after a stressor is not easily interpretable towards stress-effects on immune responses (Dantzer, 1997; Dantzer and Kelley, 1989). Thus, because we measured effects of stress on several different immune parameters at many time-points after the application of the stressor, we may have been able to detect phenomena that are easily missed when one immune parameter is measured at a single time-point after the stressor. The consideration of the temporal dynamics of the immune response, as well as the stress response, are essential for a better understanding of stress-immune system interactions.

The finding that barrows suffer from immunosuppression after mixing and gilts do not, cannot be explained by differences in the level of aggression or in height of the response of adrenaline and noradrenaline after mixing. Barrows and gilts did not differ in their response to mixing for these parameters in the present study. The only weak difference between barrows and gilts was found for cortisol levels after mixing. Although we did not find the statistical proof for an interaction effect between gender and treatment on cortisol levels, a comparison between mixed barrows and mixed gilts at 1 hour after mixing showed that mixed barrows tended to have higher salivary cortisol levels than mixed gilts. This is in accordance with a study on circadian rhythmicity in salivary cortisol concentrations in pigs (Ruis et al., 1997). There, it was shown that the mean concentration of salivary cortisol around which circadian oscillations occur, was higher in barrows than in gilts. Also, barrows showed a higher amplitude in their circadian rhythm of salivary cortisol concentration at one day after isolation than gilts. But, other studies in pigs have not shown any differences between barrows and gilts in basal and stress-induced HPA-axis activity (De Jong et al., 1998; Ekkel et al., 1996b; Otten et al., 1999; Tuchscherer et al., 1998). However, based on results from other species, it might be expected that castrated males show a higher HPA-axis reactivity than intact males. In the first place, the stressful experience of being castrated may have sensitized these pigs for later stressors (Bruijnzeel et al., 1999; McGlone et al., 1993a; Raeside et al., 1997; Vandenheede and Bouissou, 1996). In the present study, castration was carried out relatively late (at 3 weeks of age), which may have strengthened the sensitization process. In the second place, it is known that a higher HPA-axis reactivity is caused by the lack of testosterone after castration. In rodents, testosterone inhibits HPA-axis reactivity to stressors.

Castrated males thus show higher corticosteroid responses than intact males (Da Silva, 1999; Gaillard and Spinedi, 1998; Handa et al., 1994a,b).

As expected, dominance status also influenced the effect of mixing on the immune response. Ex-vivo lymphocyte proliferation after vaccination was lower in mixed dominants than in control dominants, whereas mixed and control subordinates showed equal responses. This implies that dominants are more vulnerable to the effect of mixing on the immune response than subordinates. After challenge, the IFN-gamma/IL-10 ratio of the immune response was lower in mixed dominants than in mixed subordinates, whereas control dominants and control subordinates did not differ. IFN-gamma is a typical Th1-type cytokine, that is involved in protection against viral infections, whereas IL-10 is a typical Th2-type cytokine, that has anti-inflammatory properties as well as a role in allergic reactions and anti-parasitical immune responses (Mosmann and Moore, 1991). These two cytokines are known to downregulate each other, and they tend to skew immune responses towards either a cellular (Th1), or a humoral (Th2) direction. The skewing of the Th1/Th2-balance of immune responses is under neuroendocrine control, with cortisol being able to direct the immune response towards a Th2-type, and dehydroepiandrosterone (DHEA) being able to promote a Th1-type response (Chiappelli et al., 1994; Daynes and Araneo, 1992; Daynes et al., 1990. Thus, the ratio between IFN-gamma and IL-10 production reflects the Th1/Th2-balance of the immune response, and is therefore an important aspect of the functional outcome of the immune response. A lower IFN-gamma/IL-10 ratio, as was seen in mixed dominants, reflects a Th2-skewed immune response, which is less adequate for protection against viral infections. Indeed, mixed dominants excreted more virus after challenge than mixed subordinates did.

Previous research has also shown that stress and dominance status interact in their effect on the immune system. In a series of studies it was shown that heat and shipping stress differentially affect leucocyte numbers, natural killer cell activity and mitogen-induced lymphocyte proliferation in dominant, intermediate and subordinate pigs (Hicks et al., 1998; McGlone, 1990; McGlone et al., 1993b; Morrow-Tesch et al., 1994). Another study showed that mitogen-induced lymphocyte proliferation increased in dominant pigs after mixing, whereas it decreased in subordinate pigs after mixing (Tuchscherer et al., 1998). Whereas it seems obvious that dominance status and stress interact in their effect on the immune system, the complexity of this interaction is far from understood. The present study shows that changes in the Th1/Th2-balance of the immune response may be important in determining how stress and social status interact.

Dominance also affected immunity regardless of treatment. The lymphocyte proliferation response after vaccination was higher in dominants than in subordinates. Also, the IFN-gamma response at 20 days after vaccination tended to be higher in dominants than in subordinates, and at 6 days after challenge, dominant gilts tended to have higher IFN-gamma responses than subordinate gilts. The IgG1/IgG2-ratio of the secondary immune response (after challenge) tended to be higher in dominants than in subordinates. It thus seems that for a few immune parameters, dominant pigs show higher responses than subordinate pigs. This is in agreement with previously published data (Hessing et al., 1994c; Tuchscherer et al., 1998).

Even though castrated males were used, gender had profound influences on immune responses. A main effect of gender was found for antibody and cytokine responses at various intervals after vaccination and challenge. Gilts showed higher IgG2-responses after vaccination, and a lower IgG1/IgG2-ratio after vaccination than barrows. After challenge the IgG1/IgG2-ratio tended to be lower in gilts than in barrows. The IgG1/IgG2-ratio may reflect differences in the Th1/Th2-balance of the immune response. However, this has not been proven for pigs. In mice it is known that IgG2a is stimulated specifically by Th1-type cytokines, whereas IgG1 is stimulated specifically by Th2-type cytokines (Finkelman et al., 1990). Thus, the lower IgG1/IgG2-ratio in gilts may be interpreted as a more Th1-type response in gilts than in barrows. This is in accordance with the finding that the IFN-gamma/IL-10-ratio after challenge was higher in gilts than in barrows. It thus seems that gilts are more Th1-prone than barrows, which agrees with findings in mice (Wilcoxen et al., 2000).

We conclude that mixing of unfamiliar pigs may have long term adverse consequences for the immune response against a viral vaccine. Moreover, even long-term protection against infection with the particular pathogen may be impaired. The effect of stress on the immune response appears to be more disrupting in barrows than in gilts, and in dominants than in subordinates. Finally, the measurement of Th1/Th2-properties of the immune response seems promising with regard to the unraveling of these complex interactions in pigs.

Chapter 6

Personalities in female domesticated pigs: behavioural and physiological indications

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M.A.W. Ruis[1,2], J.H.A. te Brake[1], J.A. van de Burgwal[1], I.C. de Jong[1,2], H.J. Blokhuis[1], J.M. Koolhaas[2]

[1] Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB Lelystad, The Netherlands
[2] Department of Animal Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

Abstract

The inconclusive evidence so far on the existence of distinct personality types in domesticated pigs, led us to perform the present experiment. A total of 128 gilts from 31 sows were systematically studied from birth to slaughter in two identical trials. Intra-test consistency in individual behavioural and/or physiological reactions was studied in three different tests. We were not able to show consistencies in reactions of gilts over time to a backtest (at 2-4 days and 4 weeks of age) and to a novel environment test (at 10 and 24 weeks of age). Individual aggression, however, as measured in a group-feeding competition test in stable groups (at 10 and 24 weeks of age), proved to be highly consistent. Explanations for these discrepancies in intra-test consistencies are critically discussed. Inter-test consistencies were determined by relating the individual reactions of gilts to the backtest to various characteristics and responses to tests at a later age. The highest correlations were found when resistance in the first backtest was involved. No evidence was found for the existence of specific isolated categories of animals with respect to this resistance. For further analysis, extreme responding gilts in the first backtest (roughly the top and bottom 25% of the distribution) were classified as low resistant (LR; <3 escape attempts; n = 31) or high resistant (HR; >4 escape attempts; n = 45). By comparisons of mean responses of LR and HR gilts within groups, we have established a relationship between the backtest and several other variables. Behaviourally, the HR gilts showed more aggression in the group-feeding competition tests. Also, in the competition for the most productive teats at the anterior, a predominant position of HR piglets at this site was observed during the suckling period. The latter piglets also gained more weight during this period than LR ones. Compared to HR pigs, in the first novel environment test LR pigs hesitated longer to leave their home pens and to contact a human, but no difference in their locomotory behaviour was observed. Contrasts between LR and HR pigs in the second novel environment test were reduced or absent. Physiologically, when compared to HR gilts, LR ones had a higher reactivity of the hypothalamic-pituitary-adrenocortical (HPA) system. This was shown by higher cortisol responses to the first novel environment test, to routine weighing at 25 weeks of age, and to administration of a high dose of ACTH. It is discussed that these findings for LR and HR gilts, may provide support for the existence of behavioural and physiological responses in pigs, resembling those of proactive and reactive rodents.

Introduction

Various animal studies, predominantly those in rodents, have stated the existence of basically two distinct 'personality types' within populations (Bohus et al., 1987; Benus et al., 1991). The two types of animals are classified as either having a proactive (active) or a reactive (passive) 'coping style' (for a discussion of new terms, see Koolhaas et al., 1997a, 1999). Each type has a closely associated set of behavioural and physiological characteristics. In a given challenging situation, proactive animals show more aggression, a higher general activity and a predominant sympathetic reaction (fight/flight). On the other hand, the reactive type responds more with immobility and avoidance, and a predominant parasympathetic/hypothalamic activation (conservation/ withdrawal). These individual responses are highly consistent, both in identical situations (Benus et al., 1991) and in different unrelated, i.e. in social and non-social, situations (Benus et al., 1991; Von Holst et al., 1983). Individual responses of animals may be the result of ontogenetic and learning processes, but they are at least partially determined by genetic factors (Benus et al., 1991).

Studies in domesticated pigs have demonstrated that large individual variation in behaviour and physiology exists, but attempts to categorize these animals in distinct personality types, as done for rodents, were less succesful and showed divergent results. Domestication may have (had) an important effect on the distribution of individual characteristics, by selective breeding of pigs that were more adapted to farm conditions. In many studies no correlations were found between responses to a series of different situations, consisting of both social and non-social challenges (Forkman et al., 1995; Jensen, 1994; Jensen et al. 1995a,b; Lawrence et al., 1991; Spoolder et al., 1996). Hessing et al. (1993), however, showed correlations between responses to manual restraint in a backtest and responses to social encounters. In addition, Mendl et al. (1992) observed correlations between social success in sows and various physiological measurements. More recently, Thodberg et al. (1999) have reported the existence of stable reaction patterns across situations, characterized by novelty, including social as well as non-social contexts. Some studies provide support for consistency in the reactions of pigs to similar situations (Erhard and Mendl, 1997; Lawrence et al., 1991; Spoolder et al., 1996), while others found only few indications for this (Jensen et al., 1995b). Forkman et al. (1995) showed a lack of intra-test consistency for the backtest, whereas Hessing et al. (1993, 1994b) reported a high consistency in behavioural resistance in this restraint test over time. Hessing et al. (1993, 1994b) also argued that according to the distribution in reaction patterns in the

backtest, pigs could be classified in two types, reflecting either proactive or reactive responders. These findings of subpopulations of pigs according to the backtest, with indications for proactive and reactive types of animals, could not be supported by Forkman et al. (1995). Finally, Erhard et al. (1999) argue that tonic immobility exists in pigs and that this phenomenon may predict the behaviour of pigs in different unrelated situations.

With respect to behavioural and physiological reactions of growing pigs to management-associated stress factors, individual animals may respond differently. This may indicate differences in appraisal of situations or points to a more fundamental difference in coping with the challenge. As described above, the data on this subject are conflicting and inconclusive. To advance knowledge on this subject, the present experiment aimed at portraying some important aspects of individual variation in young gilts. Since this study is part of a larger study to investigate reactions of young gilts to various challenges, only female pigs were studied. Intra-test consistencies were tested by subjecting gilts twice to three different tests. Repeatability of reactions of gilts to novel stimuli was tested by opening of the door of the home pen (to enter a novel corridor), and by a sudden human approach. Consistency in aggressive features of gilts was tested in a repeated group-feeding competition test, in which aggressive encounters were triggered by providing only small amounts of food after food deprivation. Finally, we tried to replicate results of Hessing et al. (1993), concerning classification of pigs into proactive and reactive responders according to the backtest. Besides a repetition of the test over time, we tested whether relationships exist between behavioural resistance in the backtest and other variables at a later age (inter-test consistency). However, our approach differed from that of Hessing et al. (1993), since we carried out a first backtest at a very young age (2-4 days after birth), with rather naive piglets.

Materials and Methods

All procedures involving animal handling and testing were approved by the Institutional Care and Use Committee of the Institute for Animal Science and Health (ID-Lelystad) in Lelystad, The Netherlands. Figure 1 shows the timing of experimental and routine procedures.

Experimental housing and animals

The experiment was conducted at the experimental farm 'Bantham' in Maartensdijk, The Netherlands. A total of 128 gilts (Great Yorkshire x (Great

Yorkshire x Dutch Landrace)) was used in two identical trials (batches). Gilts were selected from 14 and 17 litters for batch one and two, respectively. They were born in farrowing pens (2.40 x 1.60 m) with partly slatted concrete floors. Within one day after birth, piglets were weighed and received an ear tattoo for identification. Furthermore, between 2 and 4 days of age, eye-teeth and tails were clipped, and iron was injected. Piglets were weaned at 4 weeks of age by removing the sow. At 10 weeks of age, in each trial, gilts were selected and randomly assigned to 8 groups consisting of 8 gilts each. On average, the number of litters making up a group was 7-8, and each gilt was housed with a maximum of one litter-mate. They were relocated in fattening pens (3.20 x 2.25 m) with partly slatted concrete floors in another room, where they were housed until 25 weeks of age (age of slaughter). This room was ventilated and temperature controlled, with temperatures kept between 19 and 21^{0}C. Artificial lights were on from 06.00 until 18.00 h. Food (commercial pelleted dry diets) and water (from nipple drinkers) were available ad libitum. Besides within one day after birth, pigs were routinely weighed at 4, 10 and 25 weeks of age.

Experimental procedures		**Routine procedures**	
b	backtest	**w**	weighing
t	determination of teat order	**e**	eye-teeth and tail clipping
n	novel environment test	**we**	weaning
g	group-feeding competition test	**r**	relocation
a	ACTH challenge test	**s**	slaughter

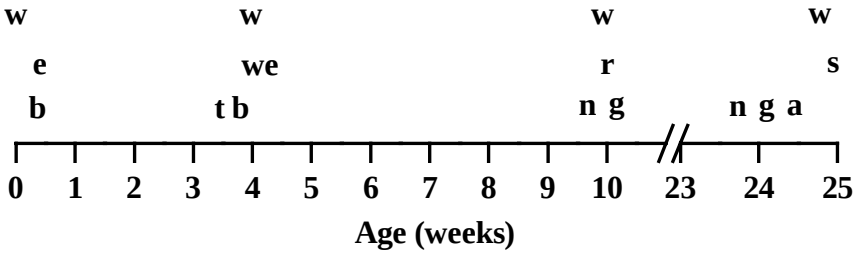

Figure 1. Timing of experimental and routine procedures.

Backtest

Each piglet was subjected to this test twice, i.e. at the start (2-4 days of age, before eye-teeth and tail clipping) and at the end (4 weeks of age) of the suckling period. The test was performed with individual piglets in a corridor close

to the home pen. A piglet was gently removed from its pen with the right arm and put on its back on a foam rubber mat. The piglet was manually restrained in this supine position for 1 min by placing the hand of the left arm loosely over the thorax of the animal (adapted from Hessing et al., 1993). The left foreleg of the piglet was held between the index and middle finger of the hand, while the piglet was allowed to move freely with the hind legs. Resistance was then determined by counting the number of escape attempts. One escape attempt was characterized by one continuous series of wriggles. The duration of escape behaviour during the test (in percentage of time) was also determined, together with registration of the number of vocalizations.

Group-feeding competition test

Animals were not allowed to eat from 12 hours before until the start of this test, carried out twice in the fattening pen, i.e at 10 and 24 weeks of age. The test, adapted from Lawrence et al. (1991), lasted 1 hour and was performed in 6 sessions of 10 min each. At the start of each session, aggressive interactions were triggered by providing a small amount of food in the front middle of the pen. It was not possible for all 8 pigs to gain access to the food at the same time. Behaviour was recorded on video and scored afterwards by using the software programme The Observer 3.0® (Noldus Information Technology, Wageningen, The Netherlands). Aggressive acts included biting (bites directed to all parts of the body), knocking (rapid thrusting the head or snout upwards or sidewards to any part of the body) and non-damaging threats (aggressive interactions, not involving physical contact). For each animal in a group, aggression was determined and expressed in an index (X) (Lee et al., 1982): $X = 0.5 \times (D - R + N + 1)$, where D is the number of other animals to which more aggressive acts were directed than received from, R is the number of other animals from which more aggressive acts were received than given to, and N = group size. The higher the index, the higher the aggression score.

Novel environment test

This test was performed twice, i.e. at the ages of 10 and 24 weeks. The first test was performed in the farrowing and the second one in the fattening room. By opening the door, pigs were allowed to leave their home pens to enter a corridor (7 x 1 m), divided in 5 equal sections and serving as an unfamiliar enclosure. The opening of the door was wide enough to allow several pigs to leave the pen simultaneously. The pigs were allowed to move freely for 10 min. At the end of this period, one experimenter suddenly entered the corridor (sudden human

approach) and remained in a stationary posture at one end of the corridor for 1 min. Behaviour was recorded on videotape and studied afterwards with The Observer® 3.0 software programme. Parameters that were determined were (1) latency time for each pig to leave the home pen; (2) locomotion in the corridor, by scoring the number of sections crossed; and (3) latency time to initiate contact with the experimenter (sniffing, chewing or biting overalls or boots). The cortisol response to the overall test was determined by sampling saliva 5 min prior to and 5 min after the test.

Teat order

The teat order of piglets was determined a few days before weaning. For each sow, functional teats were determined and teat pairs were numbered from anterior (1) to posterior (usually 7). During the first attended suckling period, piglets were given a number on the back for recognition. From the observations during 5 suckling periods, the most frequently occupied teat for each animal was determined. Only this 'primary teat' (Fraser and Jones, 1975) was used for analysis.

Routine weighing

At 25 weeks of age, routine weighing was accompanied by collecting saliva for assaying cortisol. Weighing consisted of moving individual gilts from their home pen into a mobile weighing box placed in the corridor close to the home pen. Saliva was collected 5 min prior to and 5 min after weighing.

ACTH challenge test

This test of pituitary adrenal function was performed at 24 weeks of age in the home pen of the animals. A large dose of adrenocorticotrophic hormone ($ACTH_{1-24}$, Synacthen®, Ciba-Geigy, Basle, Switserland; 200 IU per pig dissolved in 5 ml saline) was injected intramuscularly to test the capacity of the adrenal cortex under maximal stimulation. At the timepoints 5 min before and 5, 15, 30, 45, 60, 75, 90 and 120 min after ACTH administration, saliva was sampled for cortisol determinations.

Saliva collection and cortisol analysis

Both procedures were described by Ruis et al. (1997). In short, saliva samples were collected by allowing animals to chew on two cotton buds simultaneously until the buds were thoroughly moistened. The buds were placed in special centrifuge tubes and kept on ice until centrifuged for 5 min at 400 x *g* to

remove the saliva. Saliva was then stored at -20^{0}C until analysis. Cortisol concentrations were measured by using a commercial radioimmunoassay (RIA) kit (Coat-A-Count Cortisol® TKCO, Diagnostic Products Corporation, Apeldoorn, The Netherlands). The detection limit of the assay was 0.13 ng cortisol per ml, and the intra-assay and inter-assay variances were, respectively, 5,5 and 8.3%.

Statistical analysis

Spearman rank correlation coeffients (R_s) were employed to test whether individual responses to the backtest, the novel environment test and the group-feeding competition test were consistent between repeated exposures (intra-test consistency; n = 128). Initially, this correlation analysis was also performed to describe the relationship between backtest behaviour and variables from other test situations (inter-test consistency). Subsequently, as described under Results, according to backtest behaviour low resistant (LR; n = 31) and high resistant (HR; n = 45) gilts were compared for most variables.

Scores of aggression in the group-feeding competition tests were obtained per group of 8 animals (index from 1 to 8). Responses of animals to the novel environment tests were also obtained per group and were therefore dependent on the specific group. Therefore, in each group, mean scores of LR and HR animals in both tests were calculated first. Then, within-group differences between means were analyzed with Wilcoxon's signed rank test.

For associations between backtest behaviour and teat order, Chi-square analysis was used to study whether the frequencies of LR and HR pigs, suckling at the first two teat pairs (anterior; pairs 1 and 2) of the sow, differ from frequencies expected on a random basis. Similarly, frequencies of HR and LR piglets at the last two teat pairs (posterior; pairs 6 and 7) were studied and compared.

Analysis of variance was performed to analyze data with respect to cortisol responses to routine weighing, body weights (within time points), and characteristics of responses to the ACTH challenge test. Fixed effects were main effects for the factors backtest resistance and trial. Litter and group effects were introduced as random effects. Components of variance were estimated by restricted maximum likelihood (REML; Engel, 1990). Tests for main effects were based on the Wald test (Buist and Engel, 1994). Pairwise comparisons were based on a two-sample Student's t-test. Where variances of groups were unequal, an approximate t-test was used, employing an approximation of the distribution of the variance of the difference between the means (in the denominator of the test statistic), see e.g. Scheffé (1959).

For analyzing cortisol observations after ACTH administration, a separate curve was fitted to the data of each individual gilt. A family of curves based on the incomplete gamma function (sometimes referred to as Wood's curve in the context of lactation data) was employed: $Y_t = D + A \times t^B \times \exp(- C \times t) + e_t$. Here, for the individual under study, Y_t is the measured cortisol concentration at time t after ACTH administration and e_t is an associated error term. This is a unimodal curve, which starts at the baseline concentration D, increases to a maximum at $t = t_{max} = B / C$, and then decreases again to level D. B determines the rate of increase before the peak position t_{max} is reached, C determines the rate of decrease after t_{max}, and A determines the hight of the peak $Y_{max} = D + A \times t^B_{max} \times \exp(- C \times t_{max})$ at $t = t_{max}$. For each individual, its constants A, B, C and D are estimated by the method of least squares. From these individual estimates, for each animal, the area S under the curve and the slopes s_5 and s_{75} at t = 5 and t = 75 min respectively, were estimated. Each of the summary statistics t_{max}, Y_{max}, S, s_5 and s_{75} was analysed separately as a (new) response variable. Additionally, for the sake of completeness, the simple but less efficient Student's t-test was applied to observations at each individual time-point.
All calculations were performed with the statistical programming language Genstat 5® (1993). Effects were considered significant if $p<0.05$ (ns = not significant). Unless stated otherwise, data are presented as mean(±SEM). Data of both trials were comparable and no significant trial effects were observed (unless stated otherwise). Consequently, data of both trials were combined for further analyses.

Results

Intra-test consistency

There was no agreement between responses in the first and the second backtest. Rank correlations (R_s) were 0.17 (ns), 0.21 (ns) and 0.23 (ns), respectively, for the number of escape attempts, duration of escape behaviour and number of vocalizations. Moreover, no consistency was found for responses to the first and the second novel environment test: for latency to leave the home pen, locomotion in the corridor, latency to human contact, and cortisol response, correlations were –0.06 (ns), 0.21 (ns), 0.01 (ns) and –0.07 (ns), respectively. In contrast, aggression measured in both group-feeding competition tests, was found to be higly consistent (R_s=0.61; p=0.003).

Inter-test consistency: Backtest resistance versus other variables

Since backtest behaviour was not found to be consistent upon repeated testing, relationships between escape behaviour in the backtest and responses to

other tests or challenges were first studied separately for each backtest. The piglet's reactions to the first backtest were higher correlated to various other variables than reactions in the second backtest did. For instance, mean correlations with aggression were 0.33 (p=0.02) and 0.10 (ns), for resistance in the first and the second backtest, respectively. These findings led us to consider data with respect to behaviour in the first backtest, carried out at a very young age with rather naive piglets, for further analysis.

Backtest behaviour. Figure 2A shows the histogram for resistance (number of escape attempts) of individual gilts in the first backtest. The figure suggests an unimodal distribution, with no indication for a limited number of isolated classes. However, this distribution may reflect an underlying continuum in individual variation and does not exclude that animals towards the ends of the scale have a prevalence for one or another stategy. To test this, we selected gilts with extreme numbers of escape attempts (roughly the top and bottom 25% of the distribution). By this selection, we also reduced the influence of 'measurement error' in number of escape attempts (lower or higher values just by chance). On the basis of the histogram, we removed gilts in the middle two highest bars from the analysis, which seems to be a reasonable compromise with respect to the number of animals left for analysis. The two groups thus selected were entered as a two level factor in a statistical model (see statistical analysis). Piglets were classified as low resistant (LR) when less than three escape attempts were made, and as high resistant (HR) when more than four escape attempts were observed (Figure 2B). A total of 31 gilts (originating from 20 litters) was classified as LR and 45 gilts (originating from 23 litters) as HR. Table 1 shows a summary of the distribution of LR and HR gilts in the 16 groups of 8 pigs (in the fattening pens). When resistance was expressed as percentage of escape behaviour per min, a high and significant (p<0.001) contrast was also seen: 9.6±1.1 and 41.5±2.6%, for LR and HR gilts, respectively. Numbers of vocalizations were also found to differ significantly (p<0.01): LR gilts vocalized 12±3 and HR animals 32±2 times.

Table 1. Distribution of LR and HR gilts in the fattening pens.

	Trial 1								Trial 2							
Pen	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
#LR	2	2	2	3	2	2	2	1	2	2	2	2	1	2	2	2
#HR	3	3	2	3	4	3	2	3	2	3	2	3	4	2	3	3

Associations with aggression in the group-feeding competition tests. The LR gilts in each pen in the first group-feeding competition test, showed significantly (p<0.05) lower aggression towards other pigs than the HR animals in each pen: mean indexes of aggression were 3.9±0.33 versus 5.5±0.26, respectively. The contrast in aggression between LR and HR gilts was also significant (p<0.01) in the second group-feeding competition test: indexes of 3.5±0.44 versus 5.7±0.36, respectively.

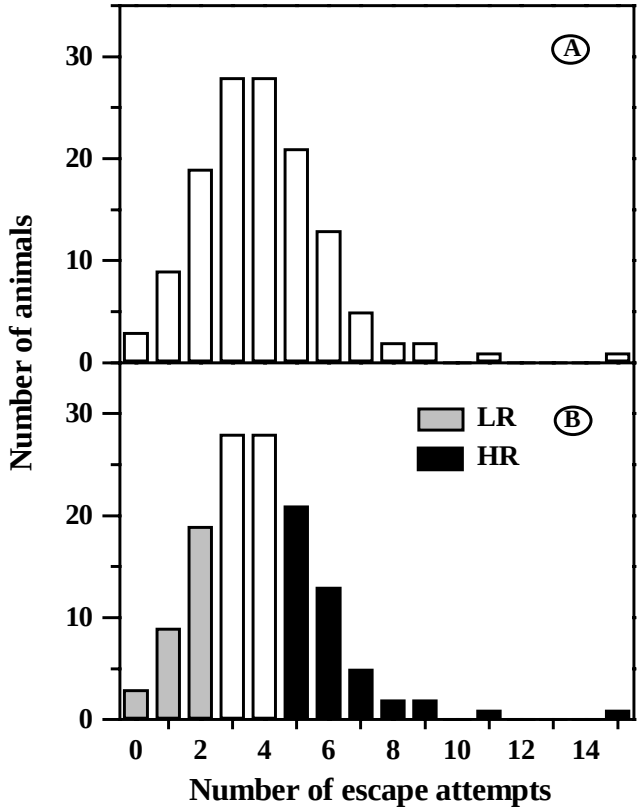

Figure 2. (A) The histogram of escape behaviour (number of escape attempts) of gilts in a 60 s backtest performed at 2-4 days of age (n = 128); and (B) The same distribution as above, but after classification of extreme responding gilts as either low resistant (LR; less than 3 escape attempts; n = 31) or high resistant (HR; more than 4 escape attempts; n = 45).

Associations with reactions to the novel environment tests. The relationships between backtest resistance and responses to the repeated novel environment test are shown in Table 2. When compared to HR gilts, LR animals showed a significantly (p<0.05) longer latency time to leave their home pen in the

first novel environment test and a tendency (p=0.05) for a longer latency time in the second one. For latencies to contact a human and for cortisol responses, significantly (p<0.05 and p<0.01, respectively) higher values were seen for LR gilts in the first novel environment test. No differences for these parameters were found in the second test. Locomotions in the corridor did not differ between LR and HR gilts in either test.

Table 2. Characteristics of responses (mean±SEM) to the repeated novel environment test for low resistant (LR) and high resistant (HR) gilts.

	Novel environment test 1		Novel environment test 2	
	LR	HR	LR	HR
Latency time to leave home pen (s)	213±22.5	131±18.1**	159±35.3	97±10.6*
Locomotion in corridor (# of sections entered)	64±13.2	77±4.5	54±8.0	55±4.9
Latency time to contact human (s)	52±3.5	37±3.4**	38±6.1	29±3.0
Cortisol response (ng/ml)	0.92±0.18	0.26±0.1***	0.73±0.25	0.47±0.12

Differences within separate tests are indicated: ***P < 0.01, **P < 0.05, *P < 0.1.

Associations with teat order. The two anterior teat pairs were significantly (p<0.05) more frequently occupied by HR gilts than expected on a random basis. The frequency data at the anterior teats for LR gilts were not found to differ from expected (random) values. The ratio LR:HR at the anterior teats was 1:3. At the two posterior teat pairs, frequency data for LR and HR did not differ from those expected on a random basis, and the ratio LR:HR gilts was 1:1.

Associations with cortisol responses to routine weighing. The cortisol response (increase) to weighing at 25 weeks of age was significantly (p<0.05) higher in LR gilts (1.07±0.11 ng/ml) than in HR animals (0.51±0.14 ng/ml). Baseline cortisol concentrations did not differ between LR and HR gilts.

Associations with cortisol responses to ACTH. When analyzing the characteristics of individual cortisol plots, backtest resistance was significantly (p<0.01) related to the height of the cortisol peak after ACTH injection, being higher for LR (6.5±0.45 ng/ml) than for HR gilts (5.1±0.35 ng/ml). Areas under the curves (AUC) were also significantly (p<0.05) higher for LR (513±37) than for HR

gilts (414±26). No differences were found for other curve variables. Figure 3 illustrates the within time differences between LR and HR gilts. Cortisol concentrations in LR gilts were significantly higher at 15 (p<0.01), 30 (p<0.05), 45 (p<0.01) and 60 (p<0.01) min after ACTH injection.

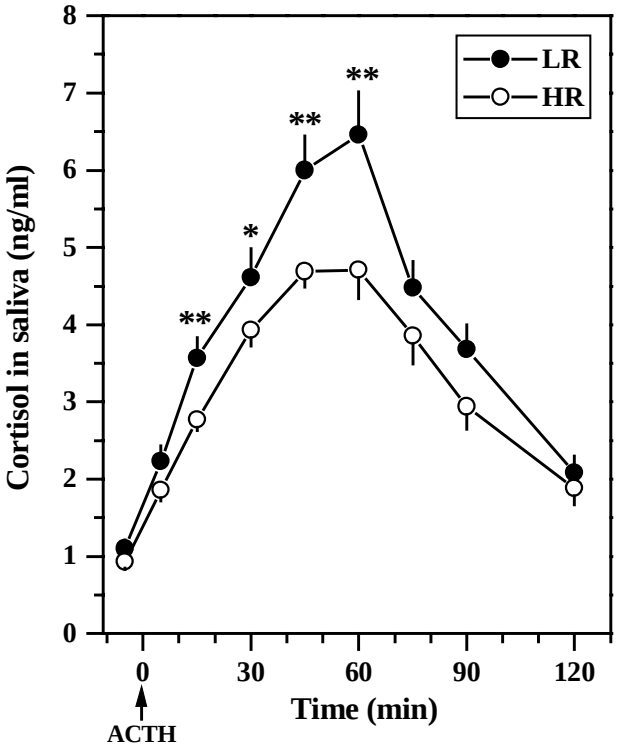

Figure 3. Mean (±SEM) salivary cortisol concentrations (ng/ml) of 45 HR and 31 LR gilts before and after administration of $ACTH_{1-24}$ (200 IU per pig; i.m. injection). Differences between LR and HR gilts within timepoints: $^{**}p$<0.01, $^{*}p$<0.05.

Associations with body weight. At 4 weeks of age, but not at birth, the difference between body weights of LR (7.21±0.29 kg) and HR gilts (8.27±0.23 kg) was significant (p<0.05). At 10 weeks of age, a tendency (p=0.09) for a higher body weight of HR gilts was observed: 33.2±0.9 versus 31.3±0.8 kg. No differences between LR and HR gilts were observed at the other ages. Differences in body weight between trials were significant at 4 (p<0.01), 10 (p<0.01) and 25 (p<0.05) weeks of age.

Discussion

Intra-test consistencies

Our results with respect to intra-test consistencies showed divergent results. While our data on individual aggression in a group-feeding competition test indicated a high consistency, we failed to show this for reactions to a backtest and a novel environment test. We propose a few explanations for these discrepancies. For example, they may be attributed to differences in stability of underlying emotions (motivations) of behavioural and physiological reactions to a certain test. Individual aggression, as the underlying emotion of behaviour in the group-feeding competition test, was higly consistent between two occasions. This high consistency may be due to the similarity in the purpose of performing that behaviour, i.e. the biological motivation to feed, associated with aggression to fulfil that need (see also next section). Erhard and Mendl (1997) showed that aggression, displayed towards intruders, is a relatively stable personality trait of individual pigs, being independent of age. Reactions in the novel environment test were not consistent on two occasions, and it was seen that responses to the second test were much reduced. This may indicate that animals may be affected by the first test, resulting in an altered motivation of responses over time to a comparable test situation. An altered underlying motivation of responses over time to comparable test situations was also observed by Erhard and Mendl (1999) and Erhard et al. (1998) for behaviour in a so called tonic immobility test. They showed that susceptibility to tonic immobility changed across different test days. Our results may reflect that when tested for the first time, latency times to leave the home pen may be a measure of fear or timidity to leave the familiar environment (Archer, 1973; see also Erhard and Mendl, 1999). However, when the test is repeated, the threatening impact of leaving the home pen may be much reduced and reactions may be a reflection of reduced fear or another (balance of) emotion(s). An increased willingness of pigs to move from their home pens voluntarily upon repeated opening of the door was also found by Geverink et al. (1998). A similar reasoning for a reduced fearfulness upon reexposure, may be followed for the shortened latency period to contact an experimenter in the human approach test. The perception of the novel environment test may also be changed by aging, i.e. with an increase in age, animals become more experienced and (unescapable) difficulties may arise in standardization of a test situation (methodological problems) when repeated. An example of the latter is that the size of an animal, possibly differing much between two occassions (especially in adolescent animals), may influence the perception of a test situation, such as a novel corridor and a

human being. This may be substantiated by results of Jensen et al. (1995b) who found that some behavioural patterns of pigs correlated well between three subsequent novel environment (open field) tests. In their study (with piglets at the age of 7 weeks), tests were only two days apart, whereas this was 14 weeks in our study. The previous arguments may also explain why the reactions of piglets differed so much between two backtests. Specific (adverse) experiences that may affect the perception of the threat, such as the backtest are those which were applied shortly after the first backtest: tail and eye-teeth clipping, and injection of iron. The non-repeatability of reactions to the backtest agrees with results of Forkman et al. (1995), but contrasts findings of Hessing et al. (1993, 1994b). To some extent these differences may be attributed to the time span between subsequent tests, being shorter in the studies of Hessing et al. (1993, 1994b).

We also address the limitations of the correlational technique as an explanation for the discrepancies in results on intra-test consistencies. The analysis used all individuals, irrespective of group-specifics. Although it may be emphasized that group dynamics should be excluded when testing individuality of animals, it is a fact in pig husbandry that animals are part of (often) non-homogenous groups. The design of our study is thus a reflection of everyday life. Nevertheless, it may be possible that differences between (dynamics of) groups may affect correlations, thereby limiting the interpretation of the data. This may especially be considered for individual responses in the novel environment test, in which group-specific processes may determine the magnitude of individual responses, and animals were part of different groups on two occassions. Individual scores of aggression (indices) in the group-feeding competition test, on the other hand, were rated (1-8) within each group, and individuals were part of the same groups on both occassions.

Inter-test consistencies

We provide some support for the validity of the backtest to gather information about differences between animals at a very early stage. The quality of a backtest at a very young age may be attributed to testing of rather naive animals, unaffected by previous experiences. The population distribution of escape behaviour in the first backtest was found to be continuous (unimodality; Figure 2). In our study, we have selected the extreme responders in the first backtest, the LR and HR gilts, to study relationships between resistance in the backtest and responses in other challenge tests. With the statistical procedure to test differences between LR and HR gilts, problems arising from relationships between animals in

the same group are circumvented. The pooled within-group contrasts between low and high responders for the backtest do not affect the validity of conclusions about the presence and nature of relationships. Although it may be argued that the differences between mean responses of LR and HR gilts do not necessarily reflect differences within a homogenous population of individuals, our design reflects routine procedures in modern pig husbandry.

The extremes, according to resistance in the first backtest, may adopt responses that resemble those observed in studies of rodent coping styles. Behaviourally, within each group, gilts classed as HR showed a higher mean score of aggression than those classed as LR. Behavioural studies in rodents have indicated that the more aggressive animals cope more actively with environmental challenges (Benus et al., 1991; Koolhaas et al, 1997a). Measures of aggression in these rodent studies were based upon measures of attack latencies in resident-intruder paradigms, mimicking aggression in a setting that conforms to what happens in the field. The common function of aggression, however, is clearly related to the establishment and defense of property, like territory and food. In our study, we have investigated two situations, in which aggression occurred to establish defense of food, i.e. in the group-feeding competition tests and in competition for teats during the suckling period. We have observed a relationship between higher resistance in the first backtest and a higher number of attacks towards others in the group-feeding competition tests. This agrees with results of Hessing et al. (1993), who also showed that the piglets they classified as resistant ones in the backtest, were mostly the aggressive animals. The piglets they classified as aggressive ones in social confrontation tests shortly after birth, were also the ones that mainly showed aggressive behaviour after mixing at 10 and again at 15 weeks of age. Contrasting results were found by Mendl et al. (1998), who were not able to predict aggression (expressed by attack latencies) from behaviour in the tonic immobility test, a test much resembling the backtest. With regard to competition for teats, we have observed the teat order. LR gilts less frequently occupied the anterior teats. Although controversial (Fraser and Jones, 1975), it is often suggested that the glands of the anterior teats produce more milk, and that piglets actively compete for these teats. Once a teat is preferred the occupying piglet usually defends it against the others. Hoy et al. (1995) showed that piglets in anterior position had the highest daily weight gain during the suckling period. Our results are in line with this, since a difference in body weight developed from birth to 4 weeks of age. Regarding their mean scores within groups, pigs classed as HR gained more weight than the LR classed ones. It seems that the HR animals took

advantage of their predominant suckling position at the anterior teats. The difference in body weight was less prevalent present at 10 weeks of age, and was dissappeared at the age of slaughter.

Furthermore, when tested for the first time in the novel environment test (with respect to mean scores within each group), those gilts classed as LR hesitated for a longer time to leave the home pen and were more inhibited to approach a human being, than those animals classed as HR. This may reflect a more reactive feature of the former animals. Studies of reactive rodents showed that their behaviour is more directed by environmental stimuli (extrinsic behavioural control), whereas the behaviour of proactive rodents is more routine-like and intrinsically determined (Benus et al., 1991). This means that upon a change in the environment, reactive animals more gradually and intensively respond to changes, whereas the reactions of proactive animals are less inhibited. To substantiate this, Hessing et al. (1994b) showed that their so called reactive pigs, were more inhibited to approach a novel object, but that they spent more time in exploring it, than proactive animals did. A more intense exploration of the corridor by more reactive animals may explain why in our study the (mean score of the) number of sections entered in the corridor did not differ between LR and HR classed gilts, although LR pigs took longer to enter the corridor. The more reactive feature of LR pigs was less evident in the second test, suggesting that for this parameter backtest resistance only reflects the behaviour evoked upon a first challenge (i.e fear-evoked: see previous section). In line with these findings, Erhard and Mendl (1999) showed that tonic immobility revealed something about the behaviour of pigs that were put in a potentially fearful (novel) environment for the first time, but not upon reexposure. In our study, regarding mean scores within their groups, those gilts classed as LR also took more time to initiate contact with the experimenter upon a sudden approach, but again this was only seen in the first test.

Physiologically, when compared within groups to animals classed as HR, gilts characterized as LR had higher mean scores of reactivity of the (hypothalamic)-pituitary-adrenocortical (HPA) system. This higher responsiveness of the pituitary adrenocortical system of LR gilts may resemble reaction patterns of the same axis observed in rodents having reactive coping stategies (Koolhaas and Oortmerssen, 1988; Koolhaas et al., 1997a). The LR gilts in our study displayed higher salivary cortisol responses to the first novel environment test, to routine weighing and to administration of a high dose of ACTH. We did not observe differences in baseline cortisol concentrations between LR and HR gilts, which contrasts findings of Hessing et al. (1994b) who reported higher resting (plasma

total) cortisol concentrations in less resistant pigs. Moreover, they found in the latter animals that the cortisol response to novelty was generally lower than in more resistant pigs, being opposite to our results. However, as they also argued in their discussion, samples were taken just before and 90 min after testing, so that it may be questioned whether the actual cortisol responses were measured in both groups, since both timepoints were so far apart. Also, they did not find a difference between backtest extremes in (plasma total) cortisol concentrations after intramuscular administration of a high dose of ACTH. Again, this may be explained by the previous reasoning that time intervals between (few) samplings were too long and did not allow to discriminate between animals. This is supported by our data, showing that after 60 min following injection of a high dose of ACTH, differences between backtest extremes in cortisol concentrations dissappeared (Figure 3). Another factor responsible for these diverging results between the mentioned studies may be attributed to the fraction of cortisol measured, i.e. the predominantly unbound fraction in saliva or the total (i.e. unbound and bound) fraction in plasma. The salivary cortisol concentration reflects the biologically active plasma unbound cortisol level and is unaffected by elevations in cortisol binding globulins (CBG), which confuse the interpretation of plasma cortisol levels (Vining et al., 1983).

Conclusions

Although selective breeding took place among domesticated pigs, we still observe large individual differences in behaviour and physiology. Our results indicate that individual aggression is a stable personality feature of gilts over time, shown in the group-feeding competition test. In contrast, we failed to show consistency in reactions to a backtest and a novel environment test. We found no indications for the existence of different categories of pigs. However, we argue that for gilts with extreme high or low resistance in a firstly performed backtest, at a very young age, relationships may exist between responses in this test and several other variables at later age. To conclude, our findings may provide support for the existence of behavioural and physiological responses in pigs, resembling those of proactive and reactive rodents.

Chapter 7

Implications of coping characteristics and social status for welfare and production of paired growing gilts

Submitted

M.A.W. Ruis[1,2], J.H.A. te Brake[1], B. Engel[1], W.G. Buist[1], H.J. Blokhuis[1], J.M. Koolhaas[2]

[1] Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB Lelystad, The Netherlands
[2] Department of Animal Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

Abstract

This paper considers the question whether knowledge on individual coping characteristics of growing pigs may be used to improve welfare and production after mixing. Gilts with either reactive or proactive coping characteristics were identified according to behavioural resistance in a backtest, respectively being low (LR) and high resistant (HR) in this test. At 7 weeks of age, several pairs of unfamiliar gilts were formed, and pairs and dominance relationships were studied over a 3-week period. The following pairs (combinations) were established: two LR gilts (LR/LR; n = 12), two HR gilts (HR/HR; n = 12), one LR and one HR gilt (LR gilt dominant: LR(d)/HR; n = 11), and one LR and one HR gilt (HR gilt dominant: LR/HR(d); n = 12). Results showed that on the day of mixing, aggression subsided less quickly and increases in body temperature were higher in LR/HR(d) and HR/HR pairs. Also, during the first week post-mixing, feed efficiency was lower and skin damage was higher in LR/HR(d) and HR/HR pairs. Mixing of two HR gilts caused highest levels of stress, indicated by greater catecholamine concentrations in urine following the day of mixing, and higher baseline levels of plasma ACTH at 1 week post-mixing. The lower tendency of gilts within HR/HR pairs to contact a novel object may present higher fearfulness. In contrast to those of LR/HR(d) pairs, responses of LR(d)/HR pairs revealed much lower levels of stress, which emphasized the importance of dominance relationships, being independent of coping characteristics of individual gilts. We speculate that in LR/HR pairs, dominant LR gilts were able to suppress aggressiveness of HR subordinates. HR or proactive gilts, however, may become aggressive when being dominant. General effects of social status, independent of combination, were also found. Compared to dominants, subordinates showed higher acute cortisol, body temperature and vocal responses to mixing. In the longer term, they showed a higher vocal and parasympathetic responsitivity towards the novel object, and their body growth was impaired. Measures not influenced by combination and social status included those of leucocyte subsets, prolactin, and average heart rates during novelty tests. To conclude, aggressive conditions in newly formed groups, and consequently welfare and production, may largely depend on coping characteristics of individual pigs, but also on dominance relationships. Accordingly, the practical value of the backtest is being discussed.

Introduction

Growing-finishing pigs may experience high levels of stress when mixed with unfamiliar conspecifics (Arey and Edwards, 1998), representing a major

welfare concern in the pig industry. Mixing usually induces vigorous fighting and much aggression to settle hierarchy positions (McGlone and Curtis, 1985; Meese and Ewbank, 1973), and may be followed by less intense aggression and social instability in the longer term (Ekkel et al., 1997; Stookey and Gonyou, 1994). Besides injuries, animals may suffer from health problems and growth retardation (Ekkel et al., 1995b; Stookey and Gonyou, 1994). It is therefore of great significance to identify the factors that influence aggression and levels of stress after mixing.

Pigs vary individually in a consistent manner in aggressive behaviour, and aggressiveness represents therefore an important personality trait of pigs (Erhard and Mendl, 1997; Ruis et al., 2000). Moreover, aggressive features of individual pigs are linked to the way of coping with (adapting to) challenges in general. Low-aggressive pigs are more reactive to environmental stimuli, i.e. they are behaviourally more inhibited when (socially) challenged. High-aggressive pigs, in contrast, are less directed by these challenges (intrinsically driven), and show a more proactive type of behavioural response (Ruis et al., 2000). Physiologically, low-aggressive pigs predominantly show a hypothalamic activation (Ruis et al., 2000), whereas high-aggressive pigs are more sympathetically dominated (Hessing et al., 1994b). Although the extremes in the population do not represent distinct categories of pigs (no bimodality), the concept of coping 'styles' is supported. Irrespective of the distribution curve, different ways of coping are based on a differential and consistent use of various behavioural and physiological mechanisms to adapt to the environment, and may vary in the same direction consistently over species (Koolhaas et al., 1999).

The present study investigated whether knowledge on coping characteristics of individual growing pigs may be used to improve welfare and production after mixing. Behavioural studies in pigs related to this subject showed an important influence of group composition. Hessing et al. (1994a) suggested that stability of newly formed groups is stimulated by mixing pigs with different coping characteristics, based on large variation in individual aggressiveness. Erhard et al. (1997), on the other hand, proposed that the better group integration is reached when pigs are mixed into groups of low-aggressive animals only. In the present experiment, gilts with specific coping characteristics were identified at a very young age by means of a backtest. It was previously shown that for pigs with extreme low or high resistance in the backtest, relationships exist between responses in this test and behavioural and physiological mechanisms to cope with environmental changes at a much later age (Ruis et al., 2000). Low resisting pigs in

the backtest generally adopt a more reactive way of coping, whereas the high resistant pigs are the more proactive copers. At 7 weeks of age, unfamiliar gilts with similar or different coping characteristics were mixed into pairs (pairwise combinations). Pairs, and dominance relationships within these pairs, were then studied for 3 weeks, by examining a broad range of variables, including behavioural and neuroendocrine patterns, and production traits.

Materials and Methods

The care and use of animals in this experiment conformed with the requirements of the Animal Care and Use Committee of the Institute for Animal Science and Health in Lelystad (ID-Lelystad), The Netherlands. Figure 1 shows the timing of management and experimental procedures.

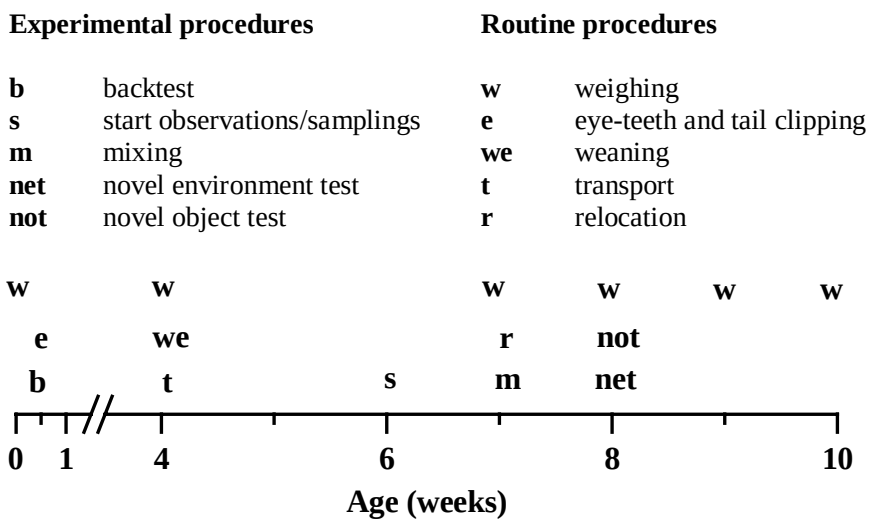

Figure 1. Timing of management and experimental procedures.

Selection of gilts with (more) reactive and proactive ways of coping

This study consisted of three identical and consecutive trials, carried out over the period January to July. Pigs (Great Yorkshire x (Great Yorkshire x Dutch Landrace)) were bred at the Experimental Farm for Pig Husbandry at Raalte, The Netherlands. They were kept in pens with half-slatted concrete floors of dimensions 3.60 by 2.20 m until weaning at 4 weeks of age. Within one day after birth, piglets were weighed and received an ear tattoo. Between 2 to 4 days of age, identification and selection took place of gilts that were likely to adopt (more)

reactive or proactive ways of coping. The identification was based on the level of behavioural resistance (escape behaviour) during manual restraint for 1 min in a backtest. On the basis of our earlier studies (Ruis et al., 2000), low resistant gilts (LR; two or less escape attempts) were considered to represent the more reactive animals, whereas high resistant gilts (HR; five or more escape attempts) were considered to be more proactive. Following the backtest, management procedures such as eye-teeth and tail clipping were carried out. Shortly after weaning, the selected gilts were brought to an experimental farm in Lelystad, The Netherlands, which is part of the Institute for Animal Science and Health (ID-Lelystad). During transport, and until the start of the actual experiment, litter-mates (3 to 5 LR and/or HR animals) were kept together and were not mixed with animals of other litters (38 litters in total).

Management and mixing procedures

Upon arrival at the experimental farm in Lelystad, groups of litter-mates were randomly assigned to one of three (adjacent) identical rooms, with constant climatological conditions. Ambient temperature was kept between 19 and 21^0C, and the light-dark cycle was 12 hour light/12 hour dark, with artificial lights on from 06.00 to 18.00 h (total lux varying from 50 to 100). Pens (2.35 x 1.70 m) had partly slatted floors and contained a nipple drinker and a food trough. Throughout the experiment, animals were given ad libitum access to water and food (commercial pelleted dry diets). Gilts were allowed to acclimatize to their housing environment and were habituated to human contact and some procedures (e.g. saliva and urine samplings, measurements of body temperature) during 2 weeks.

At 7 weeks of age, mixing procedures were started by forming pairs (pairwise combinations) of unfamiliar gilts. Formation of different pairs was done on the basis of behavioural resistance in the backtest: two LR gilts (LR/LR; n = 12); two HR gilts (HR/HR; n = 12); and one LR and one HR gilt (LR/HR; n = 24), leading to a total of 96 gilts housed in 48 pens. Gilts in each pair were weight matched and relocated in another room with pens sized 1.80 x 0.85 m with partly slatted floors. For practical reasons, within each trial, mixings were started on 4 different days, with 4 pairs of unfamiliar gilts being formed on one day. To exclude room effects, pairs of each treatment were equally divided over the three rooms. Mixings were always started between 08.00 and 10.30 h in the morning. The two pigs in each pen shared one food hopper and were not able to eat simultaneously. Pens were separated by 0.90 m high solid wooden partitions, preventing visual and physical contact between gilts of different pairs. One pair of the LR/HR

combination was later excluded from data processing due to lameness of one animal. Gilts which were not allocated to mixing procedures became part of another experiment, not being described here. These animals were socially isolated and their ways to cope with this stressor were investigated (in press: Chapter 8).

Behavioural observations

The ethogram of behaviours which were recorded is listed in Table 1. Behavioural data were collected by human observers. To minimize variability between observers, two well-trained persons did all the observations. Observations were always done during 30-min periods, in which the behaviour of each animal was scan sampled at 1 min intervals (a total of 31 observations for each 30-min period). On the day of mixing, observation periods started from time 0 of mixing and then at 30 min, 3 and 5 hours post-mixing. Additionally, behavioural observations were made on 1, 2, 7, 14 and 21 days post-mixing. On each of these observation days, behaviour was scan sampled at 1 min intervals during a 30-min period (always between 08.00 and 10.00 h). For evaluation, behavioural data were presented as a percentage of all (total) behavioural observations (except for vocalizing, which could coincide with other behaviours).

To study dominance relationships, one animal was characterized as the dominant (winner) and one as the subordinate (loser) in each pair. During the above scan-sampling periods, special (continuous) attention was given to defence-offence behaviours. An animal was considered dominant when its opponent stopped fighting and started with defensive moves. At that time the dominant was offensive by biting its opponent in the head region, particularly the ears (McGlone, 1985; Rushen and Pajor, 1987). Accordingly, the subordinate was the pig which first stopped fighting and started with defensive moves by turning away from attacks (avoidance).

Skin lesion score

At 2 and 7 days post-mixing, the body of each individual pig was examined for the frequency of all fresh cuts, scratches and wounds. The scoring included the actual number of fresh lesions, independent of size or length. Additionally, the actual length of each lesion was measured to obtain a cumulative measure of the total length of skin lesions.

Table 1. Ethogram of the behavioural measures.

Behaviour		Definition
Exploring		Rooting, sniffing, touching the pen
Inactive	Sleeping	Lying with eyes closed
	Lying	Lying with eyes open
	Sitting	Standing on fore-legs, hind quarter on the floor
	Standing	Standing inactive, may be between activities
Ingestive	Feeding	Time spent with head in the feeder and chewing feed
	Drinking	Use of water nipple to obtain water
Interactive	Manipulation	Sniffing, chewing, nosing any part of pen mate
	Display-aggression	Attacking (biting, headknocking, pushing) pen mate
Vocalizing		Total vocalizations: grunts and squeals
Walking		Walking or running through the pen

Blood, saliva and urine samplings

Blood samples (about 10 ml of blood) were taken by puncturing the jugular vein while the pig was secured with a snare. Samples were taken at time-points 2 days before, and 1 and 3 weeks after mixing, always between 09.00 and 11.00 h. They were for the greater portion transferred to prolypropylene 10-ml centrifuge tubes containing EDTA (Vacuette®, Greiner B.V., The Netherlands), and put on ice. Within 30 min, blood was centrifuged and 1.5 ml aliquots of plasma were either frozen at -20^0C (for total cortisol measurements) or at -80^0C (for ACTH and prolactin determinations). Smaller blood portions originating from samplings at the above time-points, were transferred to 5 ml centrifuge tubes containing heparin (Vacuette®, Greiner B.V., The Netherlands) and kept at room temperature. They were used within a few hours for leucocyte counts and differentiation.

Saliva samples, to determine free cortisol concentrations, were taken by simultaneous insertion of 2 veterinary cotton buds (on sticks) in the back of the mouth. The animals were allowed to chew for 1-2 min until the buds were thoroughly moistened. More detailed information on this widely used non-invasive

procedure is given by Ruis et al. (1997). On the day of mixing, samples were taken at 15 min prior to, and 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min post-mixing. Some of these samplings (at 15, 30 and 45 min post-mixing) coincided with behavioural samplings. At these time-points, behavioural observations were interrupted, leading to 1 or 2 missing behavioural samples. Further saliva sampling took place at 2 days prior to and 1, 2, 7, 14 and 21 days after mixing, always between 08.00 and 10.00 h. These samplings did not interfere with behavioural samplings. Finally, saliva was gathered in the combined novel environment and novel object test (see further). Saliva was stored at -20^{0}C until analysis.

Spontaneously voided urine samples were collected in early morning periods (between 06.00 and 08.00 h) at 2 days prior to and 1, 3, 7, 14 and 21 days post-mixing. Urine was collected in buckets (500 ml) fastened on long sticks (2 m), which allowed to catch the urine from a distance, when an animal spontaneously started to urinate. By this method, disturbance of the animals, and fecal contamination in the urine, was kept to a minimum. Samples were immediately placed at 4^{0}C and were adjusted to pH 3 within 2 hours using 6 *M* HCL. One ml aliquots were frozen at –20 ^{0}C until analysis for noradrenaline, adrenaline and creatinine. In contrast to the other samplings, urine samplings were not 100% succesful, but varied between 71 and and 95% for each combination.

Hormone and immunological determinations

Hypothalamic-pituitary-adrenal (HPA) activity. Plasma ACTH was measured according to a radioimmunoassay® procedure (RIA; Nichols Institute Diagnostics, San Juan Capistrano, USA), routinely performed in our lab (Ruis et al., 2001). The intra- and interassay CV were 3.9 and 6.5%, respectively. The detection limit was 1.0 pg/ml. Plasma concentrations of total cortisol were quantified with a time-resolved fluoroimmunoassay (TR-FIA) assay (Erkens et al., 1998; Ruis et al., 2001). Intra- en interassay CV were 6.5 and 8.1%, respectively. The detection limit was 1.6 ng/ml. Salivary cortisol was measured by using a solid-phase RIA kit (Coat-A-Count Cortisol® TKCO, Diagnostic Products Corporation, Apeldoorn, The Netherlands), modified and validated for pig salivary cortisol (Ruis et al., 1997). Intra- en interassay CV were 9.6 and 11.3%, respectively. The detection limit was 0.13 ng/ml.

Plasma prolactin. Plasma concentrations of prolactin were quantified in one assay, by means of a RIA, as previously described (Erkens et al., 1992). The intra-assay CV was 10.8% and the detection limit was 0.4 ng/ml.

Urinary catecholamines and creatinine. Urinary catecholamines (noradrenaline and adrenaline) were assayed using a high performance liquid chromatography (HPLC) procedure with electrochemical detection (Ruis et al., 2001) following a two step extraction. One hundred µl urine was extracted using the sephadex extraction described by Westerink and Koolstra (1986). This first clean-up step resulted in a 2.5 ml extract of the urine sample. One ml of this extract was taken and subjected to the liquid extraction (twice) according to the procedure described by Smedes et al. (1982). One hundred µl of the extract obtained after this second clean-up step was injected into the HPLC system. Detection limits were 35 pg/ml for noradrenaline and 55 pg/ml for adrenaline. Creatinine levels were determined using a colorimetric quantitative reaction (Boehringer PAP-method). Color intensity was measured at 510 nm. Intra- en interassay CV were 2 and 5%, respectively. To correct for variable dilutions of urine related to water intake, catecholamine levels were expressed as ratios to creatinine concentrations: noradrenaline/creatinine (NC) and adrenaline/creatinine (AC) ratios.

Leucocyte differentials. As a measure of immune function, leucocyte enumeration and differential leucocyte counts were performed in peripheral blood. Total leucocyte numbers were determined by means of an automated cell counter (Sysmex®, F-800, TOA Medical Electronics, Kobe, Japan). After staining of blood smears with a Hema-Tek slidestainer, a total of 100 cells was counted using a light microscope, and leucocytes were identified as lymphocytes, monocytes, neutrophils, eosinophils, or basophils. Because only 1 to 5% of leucocytes were eosinophils, basophils and monocytes, analysis for treatment differences was restricted to % lymphocytes and % neutrophils.

Body temperature

Body temperature was measured by means of a thermometer inserted in the ear (ThermoScan®, IRT 3020, Braun, Germany). This thermometer, which is developed for human use, measures the infrared heat generated by the eardrum and surrounding tissue. To validate this way of thermometry in pigs, a pilot was done prior to the experiment in which rectal temperatures were compared with ear temperatures. Although rectal temperatures were on average 0.6^0C higher, the correlation with ear temperature was rather high ($R_S = 0.60$; p=0.001; n = 30). At a certain timepoint, two temperature samples were taken during a period of approximately 10 s (average value used for analysis). For this purpose, a pig was held (mild restraint) by one person, while another person inserted the thermometer in the ear. Samples were taken before mixing: at –7 days and –15 min, and

following mixing, at 1, 3 and 5 hours, and on days 1, 2, 7, 14 and 21. Samples were always taken between 09.00 and 11.00 h (except for those on the day of mixing), and did not overlap with the collection of behavioural data.

Behavioural, cortisol and cardiac responses to novelty

At 1 week after mixing, two novelty tests were performed according to procedures described by Ruis et al. (2001). In a novel environment test (NET), gilts were allowed to enter a novel arena following opening of a startbox. The gilt was then allowed to explore the arena for 10 min. Behavioural parameters that were determined were the latency time to leave the startbox, and locomotion and number of vocalisations in the arena. Immediately following the NET, a novel object test (NOT; 5-min period) was performed in which gilts in the arena were confronted with a novel object consisting of a yellow and a grey bucket, tied together. The following behavioural parameters were studied: contact latency, number of contacts, total time of contact, and number of vocalizations. The cortisol response to the overall test (NET and NOT) was determined by sampling saliva 5 min prior to, and 5 and 15 min after testing.

During the NET and NOT, heart rate and time domain heart rate variability (HRV) measures were obtained via the heart rate monitor Vantage® NV (Polar Electro Oy, Kempele, Finland), which allows to determine beat-to-beat (R-R) intervals. The following parameters were quantified, according to Sgoifo et al. (1999): (1) mean heart rate (beats per minute: bpm), as measured from R-R interval durations (RR, ms); (2) overall HRV (sympathetic-parasympathetic autonomic balance), as estimated by (a) the standard deviation of the mean RR (SD, ms) and (b) the ratio between the standard deviation of the mean RR and the mean RR (SD/RR, coefficient of variance); and (3) parasympathetic influence on HRV, as expressed by the root mean square of successive RR differences (r-MSSD, ms). Values were quantified for the first min and total duration of the NET and NOT.

Growth, feed intake and gain/feed ratio

Pigs were weighed shortly before the start of mixing and weekly thereafter. Feed intake was calculated by keeping a daily record per pen of all feed added to, and the weight of, the feed hoppers. From these data, feed intake, live-weight gain, and gain/feed ratio were calculated per week.

Statistical analysis

Means of pairs were analyzed by analysis of variance with main effects for trial and combination. For analysis of percentages a logistic regression model was employed with a multiplicative overdispersion factor. The analysis was based on maximum quasi likelihood (McCullagh and Nelder, 1989). Effects for expected fractions p (= percentage / 100) were introduced on the logit scale:

logit(p) = log(p / (1-p)) = sum of main effects for trial and combination.

A variance function of the form c*p*(1-p) was assumed, where the unknown dispersion factor c was estimated from Pearson's chi-square statistic. Similarly, counts were analyzed as overdispersed Poisson data on a logarithmic scale, i.e. for expected counts m:

log(m) = sum of main effects for trial and combination,

and a variance function c*m was assumed. Latency times were analyzed on a logarithmic scale as well, again with variances assumed to be proportional to the means. For the number and length of skin lesions following mixing, linear and quadratic covariables for levels prior to mixing were included in the model. Pairwise comparisons were made employing *t*-tests with a pooled dispersion estimator.

For analysis of the effect of social status for continuous variables, such as hormone concentrations, differences $y_d - y_s$ between the dominant (d) and subordinate (s) animals within pairs were calculated and analyzed. For count data, conditional upon the total count within a pair, say $n = n_d + n_s$, the fraction n_d / n for the dominant animal was analyzed. Covariables for skin damage prior to mixing, in the comparison between dominant and subordinate gilts, did not significantly add to the model, and were therefore deleted from the model. Model and analysis parallelled the analysis of percentages. For fractions (= percentages / 100) a new fraction $z = (p_d - p_s + 1) / 2$ was evaluated and analyzed. Effects were introduced on the logit scale and equal variances were assumed. Again a maximum quasi likelihood analysis was performed. For continuous variables no effect of social status corresponds to an expected value of $y_d - y_s$ of 0, for counts and fractions the expected value of n_d / n or z is 0.5, which corresponds to value 0 on the logit scale. Effects of trial and combination represent interactions with social status. All calculations were performed with the statistical programming package Genstat 5® (1993). Differences were considered significant if $p<0.05$ and data are presented as mean±SEM.

Results

Overall, most aggressive interactions were observed in the first hour after mixing, being at much lower levels thereafter. Therefore, we give a separate description of the more acute (day of mixing) and the more long-term (from the day of mixing onwards) effects of mixing. Scores of skin lesions at 2 days after mixing were considered to result predominantly from aggressive interactions shortly after mixing, and were described in the acute effects section. Accordingly, this was also done for concentrations of urinary catecholamines on the day following the day of mixing.

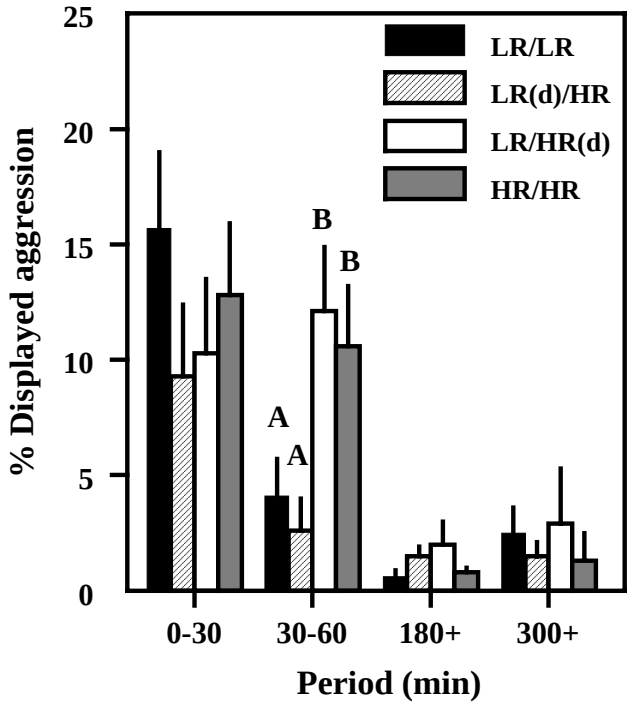

Figure 2. Mean(±SEM) percentage of displayed aggression of pairs of gilts during 30-min periods on the day of mixing. Low (LR) and high (HR) resistant gilts in the backtest were mixed in the following combinations: LR/LR (n = 12), LR(d)/HR (n = 11), LR/HR(d) (n = 12) and HR/HR (n = 12). Combination significantly affected aggressive behaviour in the second 30-min period ($p<0.05$). Means without a common superscript differ significantly ($p<0.05$).

Acute effects of mixing

Dominance relationships, aggression and skin lesions. During the first day of mixing, dominant and subordinate gilts became identified in all pairs. Because in LR/HR pairs there was an almost equal number of LR and HR dominants, we

differentiated between LR/HR pairs according to the social status of individuals: LR(**d**)/HR (LR gilt **d**ominant; n = 11) and LR/HR(**d**) (HR gilt **d**ominant; n = 12). Characteristics of aggressive behaviour (attacks directed to pen-mate) of pairs of gilts on the first day of mixing are shown in Figure 2. During the first 30 min, aggression was relatively high, with no significant effect of combination. Within-pair differences were not observed, indicative for mutual aggression (fighting). In the second 30-min period, however, combination was significantly (p<0.05) related to differences in levels of aggression, and most aggressive acts occurred in LR/HR(d) and HR/HR pairs. In this period, aggressive behaviour became less bidirectional, and most dominance relationships were established (in 10 of 12 LR/LR pairs; in 10 of 11 LR(d)/HR pairs; in 10 of 12 LR/HR(d) pairs; and in 10 of 12 HR/HR pairs). Overall, dominants displayed significantly more aggression (4.2±1.78%; p<0.05), with a relatively small difference in HR/HR pairs (difference in %: 1.2±0.68; combination x social status interaction: p<0.05). After the first hour of mixing, levels of aggressive behaviour had much declined and between-pair differences were not detected anymore. Similarly, differences in aggressive acts between dominants and subordinates were small and negligible.

At 2 days after mixing, total numbers of fresh skin lesions differed significantly (p<0.05) between the different pairs (Figure 3). Scores were highest in LR/HR(d) and HR/HR pairs, differing significantly (p<0.05 at least) from LR(d)/HR pairs. Subordinate gilts had on average more skin lesions than dominant gilts (p<0.01), but the magnitude of differences between gilts depended on the type of combination (combination x social status interaction: p<0.05; Figure 3). Similar results (patterns and differences), according to combination and social status, were obtained for total length of skin lesions (data not shown).

Other behaviours. On the day of mixing, profiles of behaviours not involving aggression did not differ between combinations. Overall, percentages of exploratory, inactive and ingestive behaviours, and vocalizing, were 18.3±1.15, 60.0±1.44, 9.8±0.85, and 7.4±0.78, respectively. Walking and manipulation behaviours were less prevalent (below 2%). Only vocalizing seemed to be affected by dominance status, as demonstrated in the second 30-min period after mixing: subordinates vocalized more often than dominants (overall difference in %: 3.1±1.21; p<0.01), but the contrast was largest in HR/HR pairs (difference in %: 6.11±1.90; combination x social status interaction: p<0.05).

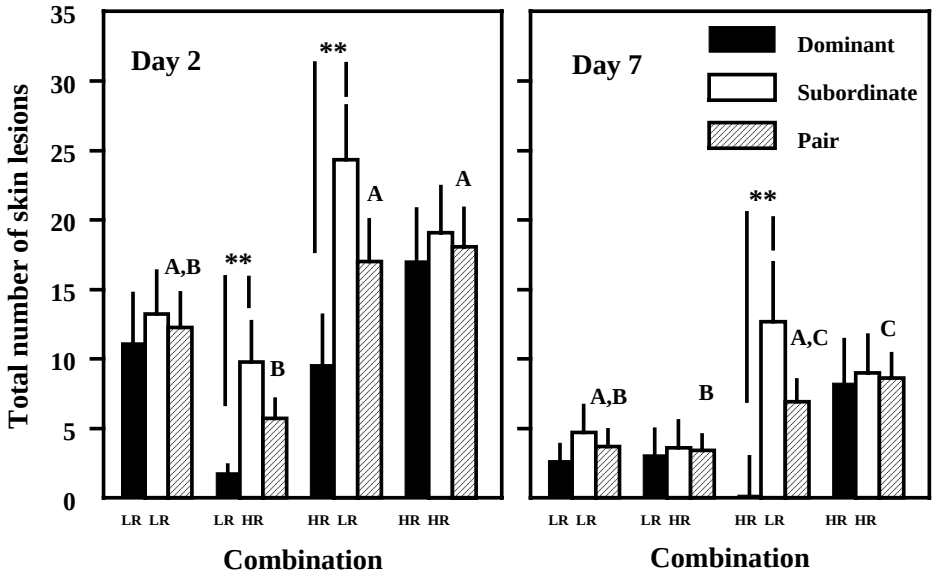

Figure 3. Total number (mean±SEM) of fresh skin lesions for pairs of gilts, and for dominant and subordinate gilts. Low (LR) and high (HR) resistant gilts in the backtest were mixed in the following combinations: LR/LR (n = 12), LR(d)/HR (n = 11), LR/HR(d) (n = 12) and HR/HR (n = 12). Skin damage was scored at 2 (left panel) and 7 (right panel) days after mixing. At pair-level, combination significantly affected scores of skin lesions at both time-points ($p<0.05$), and means without similar superscripts (letters) differed significantly ($p<0.05$). Effects of social status on skin damage were significantly related to combination at both time-points (interaction: $p<0.05$). The asterisks indicate a significant difference between dominant and subordinate gilts ($^{**}p<0.01$).

Salivary cortisol and urinary catecholamines. During the first 6 hours post-mixing, salivary cortisol responses (maximum change from baseline (baseline: average value of -2 days and -15 min) in ng/ml: LR/LR: 3.36±0.37; LR(d)/HR: 2.78±0.39; LR/HR(d): 3.45±0.37 and HR/HR: 3.54±0.37) and peak (maximum) values of salivary cortisol (in ng/ml: LR/LR: 4.83±0.36; LR(d)/HR: 4.12±0.39; LR/HR(d): 5.08±0.37 and HR/HR: 4.97±0.37) did not differ between pairs. Quantitative total responses (areas under the curves: AUC's) during this period, however, were significantly affected by combination ($p<0.05$; Figure 4), with the lowest responses in LR(d)/HR pairs. In general, total cortisol responses tended to be higher in subordinates than in dominants ($p=0.1$), and the difference was largest in LR/HR(d) pairs (combination x social status interaction: $p=0.09$; Figure 4).

Early morning urine samplings for determinations of catecholamines showed that after the first day of mixing, noradrenaline/creatinine (NC) and

adrenaline/creatinine (AC) ratios were most strongly increased (compared to pre-mixing values) in HR/HR pairs and to a much lower extent in LR(d)/HR pairs (significant effect of combination: $p<0.05$; Figure 5). The magnitude of increases in NC and AC ratios did not differ between dominant and subordinate gilts.

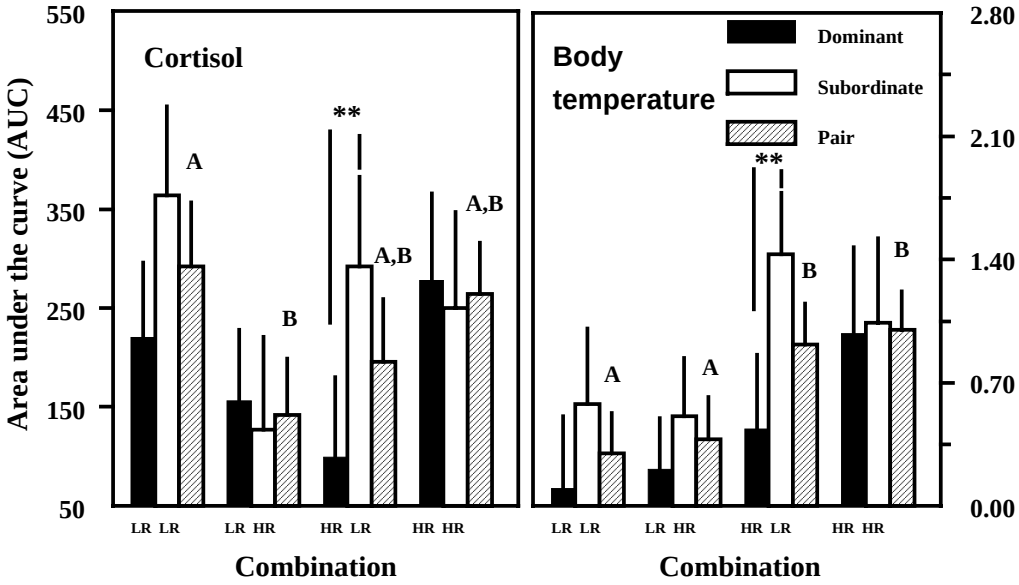

Figure 4. Total salivary cortisol (left panel) and total body temperature (right panel) responses (mean±SEM) on the day of mixing, of pairs of gilts, and of dominant and subordinate gilts. Low (LR) and high (HR) resistant gilts in the backtest were mixed in the following combinations: LR/LR (n = 12), LR(d)/HR (n = 11), LR/HR(d) (n = 12) and HR/HR (n = 12). Total salivary and body temperature responses of pairs of gilts, expressed in area under the curves (AUC), were significantly influenced by combination ($p<0.05$). Pair means without similar superscripts (letters) differ significantly ($p<0.05$). Effects of social status on total salivary cortisol (tendency for an interaction: $p=0.09$) and total body temperature (interaction: $p<0.05$) responses were depending on combination. The asterisks indicate a significant difference between dominant and subordinate gilts ($^{**}p<0.01$).

Body temperature. During the first 5 hours post-mixing, body temperature responses (maximum changes from baseline (baseline: average value of -7 days and -15 min)) were not influenced by combination (change in ^{0}C: LR/LR: 0.48±0.13; LR(d)/HR: 0.45±0.14; LR/HR(d): 0.68±0.13 and HR/HR: 0.62±0.14). Peak (maximum) body temperatures did also not differ between pairs (in ^{0}C: LR/LR: 39.48±0.14; LR(d)/HR: 39.49±0.15; LR/HR(d): 39.55±0.14 and HR/HR: 39.67±0.14). Notwithstanding, there was a significant ($p<0.05$) effect of combination on the total body temperature response (AUC's) during the above

period (Figure 4), being greatest in LR/HR(d) and HR/HR pairs. Overall, body temperature responses were higher in subordinates (difference in ^{0}C: 0.20±0.06; p<0.01) than in dominants, being most pronounced within LR/HR(d) pairs (difference in ^{0}C: 0.30±0.10; combination x social status interaction: p<0.05). A similar pattern was observed for total body temperature responses (Figure 4).

Long-term effects of mixing

Behaviour and skin lesions. Beyond the first day of mixing, no relationships were found between combination and levels of aggressive behaviour. Observed incidences of aggressive acts were rather low (overall percentage: 1.48±0.21%), and differences between pigs of different social status were not significant. Indirect assessments of levels of aggression, however, by scores of fresh skin lesions, indicated that gilts in HR/HR pairs were more involved in aggressive interactions in the first week after mixing (significant effect of combination: p<0.05). At the end of this week, these pairs displayed more injuries than LR/LR and LR(d)/HR pairs (p<0.05 at least; Figure 3). Subordinates showed on average twice as much injuries than dominants (overall effect of social status: p<0.05), but this effect was mainly caused by a large contrast within LR/HR(d) pairs (combination x social status interaction; p<0.05; Figure 3). Profiles of total length of skin lesions followed those of numbers (data not shown).

Neither combination, nor social rank, did have any effect on other behavioural parameters, at any of the 30-min sampling periods. During the 3-week period, average percentages for pairs of exploration, inactivity, ingestion, manipulation, walking and vocalizing were 8.8±0.79, 71.8±2.35, 9.6±0.86, 2.9±0.58, 0.7±0.10, and 5.7±0.61, respectively.

HPA-axis activity, prolactin and immunological characteristics. Beyond the day of mixing, salivary cortisol concentrations were similar between the different pairs, and were not different from pre-mixing values: they ranged between 0.85±0.15 and 1.33±0.25 ng/ml. Differences between dominants and subordinates in salivary cortisol were also not observed. An influence of combination on changes in blood variables was only found for ACTH (Table 2). Plasma ACTH concentrations were relatively high in HR/HR pairs, compared to the other pairs. For all plasma variables, dominant and subordinate gilts did not differentially contribute to the mean pair values (Table 2).

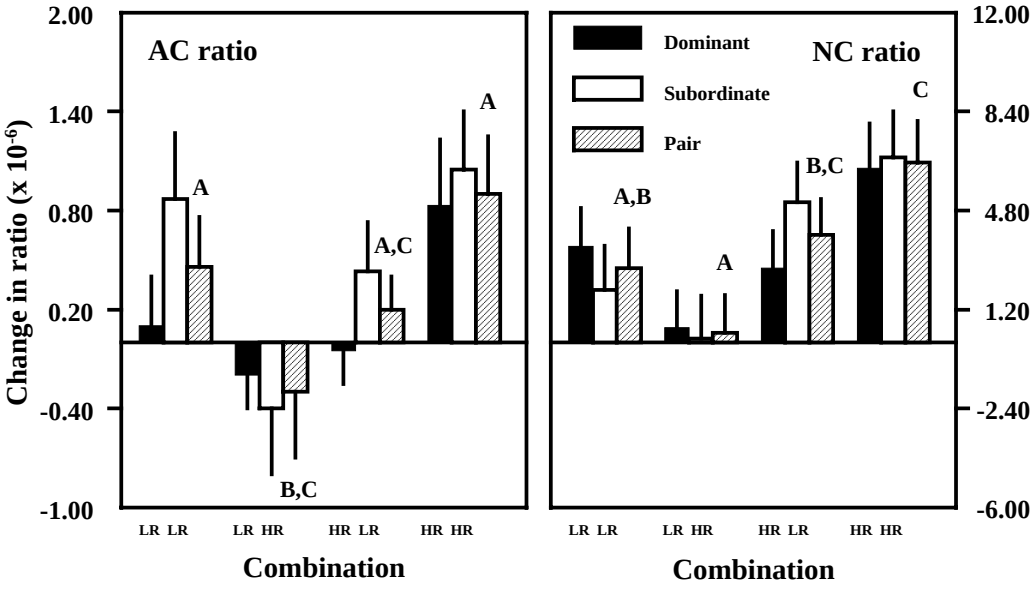

Figure 5. Mean(±SEM) catecholamine levels in urine after the day of mixing, in pairs of gilts, and in dominant and subordinate gilts within these pairs. Noradrenaline and adrenaline are expressed relative to creatinine concentrations: NC and AC ratios, respectively. Low (LR) and high (HR) resistant gilts in the backtest were mixed in the following combinations: LR/LR (sample size: n = 11), LR(d)/HR (sample size: n = 10), LR/HR(d) (sample size: n = 10) and HR/HR (sample size: n = 9). At pair-level, NC and AC ratios were significantly influenced by combination (p<0.05). Pair means without similar superscripts (letters) differ significantly (p<0.05). NC and AC ratios did not differ between dominant and subordinate gilts.

Urinary catecholamines. Analyses of NC and AC ratios showed that from the day of mixing onwards, changes in catecholamine concentrations did not differ according to combination. Relative to pre-mixing values, average NC ratios were 2.7±0.69, 0.29±0.41, -0.92±0.38, and –1.06±0.29, respectively, on day 3, 7, 14, and 21 day post-mixing. These values were –0.08±0.11, -0.01±0.08, 0.08±0.15, and –0.33±0.15, respectively, for changes in AC ratios. At all sampling points, differences in (changes in) NC ratios between dominants and subordinates depended on the type of combination (combination x social status interaction: p<0.05). Whereas differences (dominants minus subordinates) in NC ratios were rather small (between -3.05±2.08 and 1.58±1.66) in LR/LR, LR(d)/HR and LR/HR(d) pairs (no differences between these pairs), they were between 6.54±2.68 and 9.69±2.67 in HR/HR pairs (differing significantly from the other pairs: p<0.05 at least). Changes in AC ratios were not influenced at all by social status (data not shown).

Table 2. Changes in plasma hormone concentrations and percentages of circulating leucocyte subsets (mean±SEM), in relation to combination and social status.

Variable		Combination							
		Day 7				Day 21			
		LR/LR (n = 12)	LR(d)/HR (n = 11)	LR/HR(d) (n = 12)	HR/HR (n = 12)	LR/LR (n = 12)	LR(d)/HR (n = 11)	LR/HR(d) (n = 12)	HR/HR (n = 12)
ACTH	Pair[1]	-36.6±15.0^{A}	-3.3±15.7^{A,B}	-32.1±15.0^{B}	13.8±14.9^{B}	-31.5±13.1	-21.7±13.7	-36.8±13.1	-13.5±13.1
(pg/ml)	Dom-sub	19.6±39.8	-18.5±39.9	42.6±39.9	-40.3±38.2	24.6±32.5	9.50±32.4	47.6±32.4	-32.1±31.0
Cortisol	Pair	0.53±3.2	5.04±3.4	4.07±3.2	1.93±3.3	-1.22±3.3	5.75±3.2	3.52±3.0	3.22±2.9
(ng/ml)	Dom-sub	7.50±5.8	-4.29±5.6	-1.0±5.8	6.96±5.6	7.76±5.3	-2.37±5.4	6.22±5.4	3.96±5.1
Prolactin	Pair	0.04±0.2	-0.19±0.2	0.02±0.2	0.24±0.2	0.16±0.1	-0.14±0.2	0.46±0.2	0.23±0.2
(ng/ml)	Dom-sub	-0.08±0.4	-0.31±0.4	0.45±0.4	0.05±0.3	0.33±0.4	-0.33±0.4	0.47±0.4	-0.09±0.3
Lympho-	Pair	-4.15±2.7	-0.98±2.9	-0.98±2.7	-4.48±2.7	0.08±3.0	-3.88±3.1	-2.08±3.0	1.63±3.0
cytes (%)	Dom-sub	-3.11±4.8	-3.88±5.0	-2.11±4.8	-1.15±5.0	-1.08±5.7	-1.17±5.7	-4.87±5.5	-5.26±5.6
Neutro-	Pair	4.60±2.7	1.59±2.9	0.18±2.7	4.89±2.8	-1.21±3.0	3.41±3.1	3.46±3.0	-1.12±2.9
phils (%)	Dom-sub	2.06±4.9	3.39±5.1	2.06±4.9	1.39±5.1	-0.20±5.7	0.80±5.7	1.30±5.5	3.98±5.7

Values at 7 and 21 days were compared with those at 2 days prior to mixing. Pair: pairs (combinations) of gilts. Dom-sub: dominants minus subordinates (difference). [1]Significant effect of combination at 7 days after mixing ($p<0.05$). ^{A,B}Means without a common superscript differ significantly ($p<0.05$).

Body temperature. After the day of mixing, body temperatures of the four combinations did not differ significantly, and fluctuated between 38.73 and 39.06^{0}C. Also, social rank did not have any significant effect on this variable (largest overall difference in ^{0}C: 0.06±0.05, 1 day after mixing; p=0.49).

Behavioural, cortisol and cardiac responses to novelty. Of the behavioural variables studied in both novelty tests, only total time of contact in the NOT was significantly affected by combination (Table 3). Gilts of HR/HR pairs spent relatively short time in contacting the novel object. Compared to dominants, subordinate gilts generally vocalized more: 126±6.5 vs. 99±5.9 times (overall effect of social status: $p<0.05$; Table 3). Both combination and social status did not influence salivary cortisol responses to overall testing (NET + NOT; Table 3).

Heart rate and time domain parameters of heart rate variability in the NET were neither influenced by combination, nor by social status (Table 4). In the NOT, however, the SD of RR intervals was significantly ($p<0.05$) elevated in HR/HR pairs compared to LR/LR and LR/HR(d) pairs (significant effect of combination: $p<0.05$). The other measure of RR variability, i.e. the coefficient of variance (SD/RR), was not significantly influenced by combination, and this also applied for average heartrate and parasympathetic activity (r-MSSD). Significant variation in the latter parameter, however, was observed when dominant and subordinate gilts were compared in the NOT. Overall, the r-MSSD was 0.64±0.37 ms higher ($p<0.05$) in lower ranking gilts, but only during the first min of the NOT. Social status did not significantly affect average heart rate and measures of total variance in heart rate.

Production parameters. Analyses of production parameters of pairs of gilts showed a significant ($p<0.05$) effect of combination for gain/feed in the first week post-mixing, but not in the following weeks, and averaged over the whole 3-week period (Table 5). Body weight gain and feed intake of pairs were not influenced by combination. Regarding characteristics of individual gilts, a highly significant ($p<0.01$) overall effect of social status on body growth was found: during the 3 weeks following mixing, body weight gain was higher in the dominant gilts (difference in kg: 1.47 ± 0.47).

Table 3. Effects of combination and social status on behavioural and cortisol responses (mean±SEM) to novelty tests at 1 week post-mixing.

Variable		Combination			
		LR/LR (n = 12)	LR(d)/HR (n = 11)	LR/HR(d) (n = 12)	HR/HR (n = 12)
NET					
Latency to enter (s)	Pair	52.0±13.7	42.0±13.1	18.1±8.1	60.5±14.8
	Dom-sub	-13.5±18.7	-2.5±18.6	-2.1±18.6	-8.3±18.7
Locomotion (m)	Pair	102±7.7	97±8.0	115±8.2	97±7.5
	Dom-sub	8±13	-1±14	14±12	12±13
Vocalizations (number)	Pair	138±22	120±22	158±24	139±22
	Dom-sub	-6±39	28±38	-45±39	-48±39
NOT					
Contact latency (s)	Pair	42.2±12.1	41.6±12.5	29.9±10.2	39.5±11.7
	Dom-sub	-16.4±19.5	-3.8±19.3	-3.4±19.8	-7.0±19.5
Number of contacts	Pair	10.0±1.0	9.8±1.1	10.9±1.0	8.9±0.9
	Dom-sub	1.0±2.0	-1.6±2.0	-1.6±2.0	1.2±2.0
Contact time (s)	Pair[1]	43.1±6.3^{A}	47.1±6.9^{A}	39.9±6.1^{A,B}	28.1±5.1^{B}
	Dom-sub	6.6±11.9	-4.0±12.0	-14.2±12.0	4.2±11.8
Vocalizations (number)	Pair	109±11	97±10	123±12	118±11
	Dom-sub[2]	-16±18.1	-12±18.2	-50±18.0	-28±18.1
NET+NOT					
Cortisol response (ng/ml)	Pair	2.74±0.3	2.33±0.3	2.53±0.3	2.39±0.2
	Dom-sub	0.19±0.6	-0.44±0.6	0.12±0.5	0.38±0.5

NET: novel environment test. NOT: novel object test. Pair: pairs (combinations) of gilts. Dom-sub: dominants minus subordinates (difference). [1]Significant effect of combination ($p<0.05$). Means without a common superscript in the same row differ significantly ($p<0.05$). [2]Significant overall difference between dominant and subordinate gilts ($p<0.05$).

Discussion

Aggression

Immediately following mixing, the likelyhood of fighting was not influenced by individual coping characteristics of gilts, as shown by similar levels of aggressive acts in pairs of different composition. However, levels of aggressive behaviour subsided more quickly in LR/LR and LR(d)/HR pairs, compared to LR/HR(d) and HR/HR pairs. The latter two pairs also showed higher acute (total) rises in body temperature, which may indicate higher stress levels (De Jong et al., 1998), due to the more severe aggressive conditions in these pairs. After the first hour of mixing, however, aggression was strongly diminished in all pairs, which concurs with several other observations of mixed pigs (Friend et al., 1983; Rushen, 1987). Although not reflected in body temperatures, other measures indicated that pigs in LR/HR(d) and HR/HR pairs remain in a higher stressful state beyond the day of mixing. In the first week post-mixing (but not thereafter), feed efficiency as reflected by gain/feed ratio, was lowest in LR/HR(d) and HR/HR pairs, compared to the other pairs. It can be argued that the LR/HR(d) and HR/HR pairs met higher energy demands, which were, however, not significantly interfering with body growth. Because behavioural activities did not differ between pairs, it is more likely that stress was involved in this effect on feed efficiencies (Stookey and Gonyou, 1994). Animals in LR/HR(d) and HR/HR pairs, especially in the latter ones, also suffered from more skin damage. Because this was also observed at 7 days post-mixing, incidences of aggressive acts, previously shown to correlate with lesion scores (Barnett et al., 1992), seemed to persist for a prolonged time, indicative for unstable social relationships (social instability).

Stress and fear

Most obvious negative effects of mixing were observed when two HR gilts were mixed. Besides the above mentioned indications for relatively poor welfare, other variables additionally emphasize highest levels of stress and fearfulness in these pairs. Both urinary noradrenaline/creatinine and adrenaline/creatinine ratios are validated measures of sympathetic nervous system (SNS) activity (Hay et al., 2000) and were typically higher in HR/HR pairs 1 day after mixing, indicative for the greatest stress responses to the mixing procedure (Otten et al., 1999; Ruis et al., 2001). Specifically, adrenaline production is believed to be associated with increased mental stress, whereas elevations in noradrenaline also may represent a higher physical activity (Otten et al., 1999).

Table 4. Effects of combination and social status on heart rate and heart rate variability (mean±SEM) during novelty tests at 1 week post-mixing.

			Combination							
Variable	Test		LR/LR (n = 12)		LR(d)/HR (n = 11)		LR/HR(d) (n = 12)		HR/HR (n = 12)	
HR	NET	Pair	165±3.1	*173±4.8*	161±3.2	*164±5.0*	163±3.1	*172±4.8*	163±3.2	*173±5.0*
(bpm)		Dom-sub	6.2±9.7	*8.9±10.7*	-0.5±9.5	*3.6±10.5*	4.8±11.1	*-4.1±12.1*	2.5±9.6	*-2.6±10.6*
	NOT	Pair	160±3.0	*170±3.7*	157±3.2	*170±3.9*	156±3.0	*167±3.7*	157±3.2	*164±3.9*
		Dom-sub	4.2±9.1	*7.5±9.0*	-5.9±9.5	*-5.6±8.8*	-2.2±10.3	*-0.6±9.3*	3.1±8.9	*-1.1±9.5*
SD	NET	Pair	43.1±3.0	*27.6±2.3*	40.1±3.2	*28.7±2.4*	43.8±3.0	*27.7±2.3*	41.4±3.2	*27.6±2.4*
(ms)		Dom-sub	4.9±7.5	*-3.3±7.1*	-2.6±7.5	*-3.4±7.0*	-3.1±8.7	*4.9±7.9*	-7.4±7.6	*2.4±7.0*
	NOT	Pair[1]	42.8±1.9^{A}	*41.4±3.0^{a}*	45.9±2.0^{A,B}	*45.7±3.0^{a,b}*	43.2±2.0^{A}	*42.3±2.0^{a}*	48.8±1.9^{B}	*49.1±2.9^{b}*
		Dom-sub	6.2±7.5	*-5.3±9.1*	5.3±7.8	*-1.9±8.9*	-3.0±8.5	*-5.4±9.5*	-2.7±7.4	*1.9±9.6*
SD/RR	NET	Pair	0.11±0.01	*0.08±0.01*	0.10±0.01	*0.08±0.01*	0.12±0.01	*0.07±0.01*	0.11±0.01	*0.08±0.01*
		Dom-sub	0.03±0.02	*-0.01±0.01*	0.00±0.01	*-0.01±0.01*	-0.02±0.01	*0.02±0.01*	-0.02±0.02	*0.01±0.01*
	NOT	Pair	0.11±0.02	*0.11±0.01*	0.12±0.02	*0.12±0.01*	0.11±0.02	*0.11±0.01*	0.13±0.02	*0.13±0.08*
		Dom-sub	0.02±0.08	*-0.01±0.02*	0.02±0.08	*0.01±0.02*	-0.01±0.09	*-0.01±0.03*	-0.01±0.07	*0.00±0.02*
r-MSSD	NET	Pair	0.6±0.05	*1.7±0.17*	0.5±0.06	*1.4±0.18*	0.5±0.06	*1.2±0.17*	0.5±0.06	*1.3±0.18*
(ms)		Dom-sub	-0.3±0.19	*-0.8±0.56*	0.1±0.17	*-0.5±0.55*	-0.3±0.20	*0.4±0.54*	-0.2±0.17	*-0.6±0.56*
	NOT	Pair	0.9±0.11	*2.2±0.36*	0.8±0.12	*2.0±0.38*	0.8±0.10	*1.9±0.35*	0.9±0.12	*2.6±0.38*
		Dom-sub[2]	-0.3±0.30	*-1.2±0.86*	0.5±0.33	*0.4±0.84*	-0.3±0.36	*-0.8±0.99*	-0.1±0.30	*-0.8±0.90*

Average heartrate (HR), standard deviation (SD), coefficient of variance (SD/RR) and root mean square of successive RR differences (r-MSSD) during the NET (novel environment test; total 10-min period (first min in italics)) and NOT (novel object test; total 5-min period (first min in italics)). Pair: pairs (combinations) of gilts. Dom-sub: dominants minus subordinates (difference). [1]Significant effect of combination (p<0.05). [2]Significant overall difference between dominant and subordinate gilts (only in the first min; p<0.05). ^{A,B} and ^{a,b}means without common superscripts within the same row differ significantly (p<0.05).

Beyond the day of mixing, no further differences in levels of urinary catecholamines between pairs were detected, suggesting that more prolonged (chronic) stress differences did not exist. However, relatively high baseline plasma ACTH concentrations in HR/HR pairs were found at 1 week post-mixing (not thereafter). Increments in HPA activity are often associated with increased stress levels, also known to occur when pigs are socially challenged (Arnone and Dantzer, 1980; Otten et al., 1999; Ruis et al., 2001). The other feature of HPA activity, i.e. adrenocortical activity (total and free cortisol), was not variable. This suggests a physiological adaptation at the level of the adrenal glands, possibly due to a desensitisation of the adrenals to ACTH (Ladewig and Smidt, 1989). Percentages of leucocyte subsets and prolactin levels, known to be affected under conditions of social stress (Ruis et al., 2001), did not differ between the pairs. In contrast, differences existed in stress-reactivities to the specific novelty stress of the NOT, 1 week post-mixing. Gilts of HR/HR pairs typically vocalized more during the test and spent less time in contacting the novel object than the other gilts. Although vocalizing cannot unequivocally be interpreted as measures of fear (Ruis et al., 2001), lower inclinations to contact a novel object may indicate a higher level of fearfulness (c.f. Hopster et al., 1999). Behavioural variables in the novel environment test (NET) did not at all discriminate between pairs, which is in accordance with our previous thoughts that fear is not the only factor to determine responses to this test (Ruis et al., 2001). For instance, locomotory behaviour may be a reflection of both fear and exploratory motivations. In line with this, in a novelty test comparable to our NET, Andersen et al. (2000b) were not able to demonstrate anxiolytic-like effects of benzodiazepines on pig behaviour. Finally, some differences between pairs in the autonomic regulation of heart rate emerged in our study. Overall heart rate variability, quantified by the standard deviation of the mean RR, was significantly elevated in HR/HR pairs in the NOT (not in the NET), as compared to the other pairs. However, we cannot unambiguously interprete these findings in terms of balance between sympathetic and parasympathetic activities (sympathovagal balance). The r-MSSD's, specifically quantifying parasympathetic activities (Sgoifo et al., 1999), and average heart rates, did not differ between pairs. Accordingly, one might argue that the sympathetic tone is reduced, but there is no evidence to conclude this, particularly because direct assessments of sympathetic actvity, i.e. of plasma catecholamine concentrations, were not obtained during the test. We therefore conclude that our measures of cardiac activities did not provide additional information on differences

in emotional distress between pairs. This also applies for the cortisol reponses to the combined NET and NOT, which were not found to differ between the pairs.

Although some of the differences may not only be related to state variables such as stress and fear, but may also reflect aspects of personality or coping (Koolhaas et al., 1999), welfare seemed to be most seriously compromised in HR/HR pairs. When extrapolated to larger groups, one may argue that it should be avoided to form groups consisting of many HR pigs. Hessing et al. (1994a) suggested that group integration is slowed down when pigs with similar coping characteristics, and hence similar aggressiveness are mixed. We were only able to demonstrate this for paired HR pigs. Erhard et al. (1997) suggested that it is preferable to mix only low-aggressive animals. We could only partially substantiate this for LR/LR pairs, which were generally 'intermediate'. In contrast to bringing together many low-aggressive pigs, mixing pigs with different aggressive features is of much more practical relevance for pig husbandry. In the next paragraph we describe that it is very well possible to create socially stable pairs of LR and HR gilts, also indicated by Hessing et al. (1994a) for larger groups, but this is explicitly depending on the outcome of dominance relationships.

Causality of aggression: relationships with proactivity and social status

In LR/HR pairs, levels of aggressive interactions and consequently social stabilities strongly depend on the social status of LR and HR gilts. The most stable social relationship existed between a dominant LR and a subordinate HR gilt. In this situation, aggression decreased rather quickly and was predominantly observed in the first 30 min after mixing. Moreover, acute HPA-axis and SNS responses to mixing were lowest compared to the other pairs. In contrast, when a HR gilt became dominant, a less favourable social situation existed (see above). These findings essentially address the question how coping characteristics and social status may influence aggression and stabilities of social relationships when unacquainted pigs are mixed. One important difference between proactive and reactive animals is the difference in the individual's propensity to start offensive encounters. As shown in rodents, aggressive or proactive animals generally take the initiative to attack others, whereas low-aggressive or reactive animals only respond with aggression when absolutely necessary (Koolhaas et al., 1999). Individual variation in proneness to behave aggressively is also observed in pigs (Erhard et al., 1997). We therefore hypothesize that in LR(d)/HR pairs, characterized by a fast decline in levels of aggression, this behaviour was limited to initial fighting to settle disputes for social hierarchy positions.

Table 5. Effects of combination on production characteristics of pairs of gilts (mean±SEM) during 3 weeks post-mixing.

Variable	Period	Combination			
		LR/LR (n = 12)	LR(d)/HR (n = 11)	LR/HR(d) (n = 12)	HR/HR (n = 12)
Feed intake, kg	Week 1	16.67±0.98	16.90±1.03	16.49±0.98	16.97±0.98
	Week 2	19.16±0.99	19.49±1.03	19.27±0.99	19.15±0.99
	Week 3	21.78±1.15	22.51±1.20	22.19±1.15	21.94±1.15
	Total period	57.61±2.92	59.10±3.11	57.95±3.06	58.06±2.92
Weight gain, kg	Week 1	14.48±0.85	14.59±0.89	13.70±0.85	14.05±0.85
	Week 2	12.60±0.79	12.30±0.82	12.54±0.79	12.22±0.79
	Week 3	13.77±0.85	15.08±0.89	13.54±0.85	13.35±0.85
	Total period	40.85±1.82	41.97±1.91	39.78±1.82	39.62±1.82
Gain/feed, kg/kg	Week 1	0.87±0.02^{A}	0.87±0.02^{A}	0.82±0.02^{B}	0.83±0.02^{B}
	Week 2	0.65±0.08	0.63±0.08	0.65±0.07	0.64±0.08
	Week 2	0.62±0.09	0.66±0.10	0.60±0.09	0.60±0.09
	Total period	0.71±0.02	0.70±0.03	0.69±0.03	0.68±0.02

[1]Significant effect for combination (p<0.05). Means without a common superscript in the same row differ significantly (p<0.05).

This implies that although LR gilts were reluctant to start aggression they nevertheless were able to fight back properly when being attacked. Indeed, in the present study, both types were equally able to acquire the dominant position, which indicated that coping characteristics of individual pigs were of no prediction of social dominance status. When a LR gilt became dominant, it's basically low intention to use aggression may have suppressed aggressive intentions in the subordinate HR gilts, especially when the latter animals assessed their chance of winning as being low (Rushen and Pajor, 1987). In LR/HR(d) pairs, on the other hand, inhibitions in aggressive acts that should rise from dominance relationships, were not manifested. Dominant HR gilts expressed much aggression, and subordinate LR gilts served as targets, even beyond settlements of social hierarchies. This was unequivocally demonstrated by the large contrast in fresh skin lesions at 2 days, and especially at 7 days post-mixing. Thus, aggressive behaviour by a HR gilt seemed to be related to its dominant position. The persistence of aggressive behaviour, even in the absence of any further direct

provocation to behave aggressively, may imply that in this situation HR gilts are more driven by impulsive and intrinsic aggression. This is in accordance with another feature of proactivity, i.e. a behaviour which is rather independent from actual environmental stimuli. Also, proactive animals have generally a high demand to control the environment, being manifested by an aggressive mode of behaviour of top ranking HR gilts.

Our findings may thus indicate that from a welfare perspective, the situation is beneficial when reactive animals prevail among the top positions in social rank. Although it may be difficult to control the outcome of social fighting, variations in body weight may have some predictive potential. Although it is still common practice to match pigs for weight upon mixing, less aggression is observed and dominance relationships are more quickly established between pigs of different weight, with the larger animals having an advantage to become dominant (Andersen et al., 2000a; Rushen, 1987). When combined with our findings, this advantage for pig welfare by providing variation in weight may especially arise when the larger pigs have more reactive features and the smaller ones are more proactive, thereby increasing the chance that (a) reactive pig(s) become(s) highest in social rank.

Finally, the survey of the onset and persistence of aggression may be extended by the observations on gilts within HR/HR pairs. Contrary to LR/HR(d) pairs, the relatively high level of aggressive acts was bidirectional and was equally displayed by the two animals. We argue that the hostility and aggression of dominant HR gilts triggers aggressive inclinations in HR subordinates. In this case, the subordinate pigs were likely to retaliate against the dominant pigs. Evidence of aversively stimulated aggression was given in humans as well as in animals (Berkowitz, 1989), including the pig (Arnone and Dantzer, 1980), and may be provoked in situations such as ‘irritation’, ‘annoyance’, ‘frustration’ and physical pain. Although this phenomenon is widespread (Berkowitz, 1989), we believe that in accordance with the above description of proactive individuals, particularly HR gilts are vulnerable to these negative emotions, leading to aggressive acts. As a consequence of retaliation of HR subordinates, also dominant HR pigs may be negatively affected, which may explain their relatively high SNS activity, being reflected in noradrenaline (but not adrenaline) levels beyond the first day of mixing.

Dominance relationships

Despite the differences in overt aggression and social stabilities between the different pairs, general effects of social status on several measures were observed, with the impact of mixing being most pronounced in subordinate gilts. Although SNS activities did not differ between dominants and subordinates on the day of mixing, which was also shown by Fernandez et al. (1994) following social encounters, other measures elucidated higher stress responses in subordinates. This was presently shown by relatively high cortisol, body temperature and vocal responses in the subordinate animals. This may be related to the loss of social encounters (social defeat), known to result in high levels of social stress (Ruis et al., 2001). It seems likely that the adversity of social defeat is primarily related to the unpredictability and uncontrollability of the stress (Tuchscherer et al., 1998). Social rank also caused differences in the longer term. When challenged in the NOT, indications were obtained for a higher level of distress in subordinates compared to dominants. The frequency of vocalizing was higher in the subordinates and their cardiac activity was characterized by an initially higher r-MSSD, and thus higher parasympathetic activity. Again, we do not know the contribution of the sympathetic branch of the autonomic nervous system. However, because social rank did not affect average heart rates, we argue for an increased sympathetic activation in subordinates, being parasympathetically antagonized. Such a maintenance in sympathovagal balance under increasing stress levels was recently also found in situations of social stress in pigs by De Jong et al. (2000). Despite variations in aggressive interactions between pairs, subordinates also had on average twice as much skin damage than dominants in the first week after mixing. However, this may particularly result from the high skin damage in LR subordinates within LR/HR(d) pairs. Finally, social rank had a pronounced effect on body growth during the 3-week period post-mixing, as characterized by a depressed body growth in subordinate gilts compared to dominants. We recently showed that body growth of defeated gilts was not impaired when gilts lost a social encounter and were subsequently removed from the dominant (Ruis et al., 2001). We therefore argue that for subordinates, the continuing presence of dominants (social cohabitation) following a social loss, leads to a persisting higher state of (psychosocial) stress. It may not be the bodily exposures as such that determine levels of stress, but also threats (Stookey and Gonyou, 1994) and visual contact (Stefanski, 1998). However, concentrations of hormones measured in the current experiment, including cortisol, did not depend on dominance status outwith the day of mixing, and may consequently not have accounted for the differences in body

growth. To conclude, differences in social status are not always found to lead to differences in levels of stress (Sachser et al., 1998), which may be related to the degree of competition for resources and possibilities to hide (McGlone and Curtis, 1985; Rushen, 1987). Increasing competition for space and food, together with a lack of hiding places, triggers more interactions between animals, and is likely to increase the vulnerability of subordinate gilts to the adverse effects of being defeated. It is likely that in the present study, reasonable competition occurred between pair-members, because only one feeder space was available in each pen. Also, space per pig was limited (0.75 m^2). Such housing and management routines, however, (still) reflect common practice in commercially held pigs, consequently representing a threat to welfare and production of whole groups through the adverse effects on subordinates.

Conclusions

Knowledge on specific individual coping characteristics, obtained from behavioural resistance in a backtest, may be used to improve welfare and production of pigs after mixing. Bringing two high resistant or proactive gilts together was most detrimental for welfare, and proactive animals are particularly aggressive when being dominant or frustrated. When regrouping pigs, farmers may thus benefit from knowledge on coping strategies of individual pigs, and the backtest may be a helpful tool for this purpose. With growing-finishing pigs, more work is needed to determine whether pigs adopt different agonistic strategies when the number of animals in a group become too large to form social hierarchies. When social relationships become weak in large groups, the backtest may have no practical value. Sows in group-housing systems, however, are usually part of smaller groups with strong social hierarchies, and here the backtest may have a high potential.

Chapter 8

Adaptation to social isolation: acute and long-term stress responses of growing gilts with different coping characteristics

Physiology and Behavior, in press

M.A.W. Ruis[1,2], J.H.A. te Brake[1], B. Engel[1], W.G. Buist[1], H.J. Blokhuis[1], J.M. Koolhaas[2]

[1] Institute for Animal Science and Health (ID-Lelystad), P.O. Box 65, 8200 AB Lelystad, The Netherlands
[2] Department of Animal Physiology, University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands

Abstract

The present experiment studied the acute and long-term stress responses of reactive and proactive prepubertal gilts to social isolation. Gilts with either reactive or proactive features were identified according to behavioural resistance in a backtest at a young age (2-4 days), respectively being low (LR) and high (HR) resistant in this test. At 7 weeks of age, 12 gilts of each type were socially isolated. Initially, isolation was stressful for both types of gilts, as shown by increased cortisol concentrations and decreased body temperatures. Moreover, both types reacted with increases in exploration and vocalizations. Stress responses to isolation, however, differed in magnitude and/or duration between LR and HR gilts, which was in line with expected reaction patterns on the basis of preferred ways of coping. The cortisol response to isolation was higher in LR gilts, and they generally showed more explorative behaviour. HR gilts seemed to be more engaged in walking/running behaviour in the first hour after isolation, they generally vocalized more, and their noradrenaline excretion in urine was higher at 3 weeks after the start of isolation. Several responses to isolation in the longer term pointed to a prolonged higher general state of stress of HR gilts. Body temperature in HR gilts, for instance, did not recover during 3 weeks of isolation, but values returned to 'normal' within 1 day in LR gilts. At 1 week of isolation, relatively high parasympathetic responsivity to novelty was observed in HR gilts, probably due to stress-related high sympathetic reactivity. A shift in percentages of leucocyte subsets, typically occurring under conditions of stress, only developed in HR gilts during isolation. Finally, gastric ulceration was found in one HR gilt, but did not occur in LR gilts. To conclude, LR and HR gilts differed in their strategies to adapt to social isolation, and especially for HR gilts, this procedure seemed to become a chronic stressor.

Introduction

Both animal and human studies have shown the existence of individual differences in cognitive appraisal of environmental stimuli. The individual's perception of the situation determines the level of aversiveness of a stimulus and whether a state of stress is induced. When a situation is perceived as a threat, individuals differ in the way they cope with the challenge. Studies in feral populations of wild house mice and the great tit indicate the existence of basically two personality types of animals: reactive and proactive ones (discussed by Koolhaas et al. (1999)). Both types differ fundamentally in their strategy to adapt to environmental conditions. Although each type may adapt succesfully to the

environment, reactive animals may have an advantage under environmental changes. From studies with rodents it is concluded that the success of specific coping responses depends upon the stability or variability of the environment (Benus et al., 1991; Koolhaas et al., 1999). Reactive animals seem to adapt more easily to variable conditions and are more flexible. Proactive animals, on the other hand, develop routines and seem to anticipate situations, which is only of advantage in predictable (stable) conditions. In domesticated pigs, similar types can be distinguished (Hessing et al., 1994b; Ruis et al., 2000), but they represent extremes within the pig population rather than being distinct categories of animals (Ruis et al., 2000).

The aim of the present experiment was to study differences between reactive and proactive prepubertal gilts in acute and long-term stress responses to deprivation of social contact, i.e. social isolation. This experiment is part of a larger study which investigates welfare problems of growing pigs that are related to (psycho)social factors in intensive pig production. The importance of having social contact with conspecifics as such is one important aspect of investigation, being related to our studies into processes of social support (Ruis et al., 2001). As for other social species (Ahmed et al., 1995; Levine et al., 1997; Parrott et al., 1987; Rushen et al., 1999b), being socially isolated is known to be highly stressful for pigs (De Jong et al., 1998; Herskin and Jensen, 2000; Ruis et al., 1997, 2001; Schrader and Ladewig). Importantly, social isolation may have consequences for the animal in the longer term. We recently showed that, compared to socially housed pigs, isolated gilts generally develop a higher state of fearfulness, and become more responsive (more vulnerable) to environmental changes (Ruis et al., 2001). Social isolation may thus be considered as a long-term stressor, being of relevance for some pigs in intensive husbandry conditions, i.e. for individually kept sows and boars, but also for (growing) pigs which are singly kept for experimental purposes. The above reasoning led us to use social isolation as an environmental challenge or change. Individual differences in appraisal and adaptation (coping) were studied, and compared with expected stress responses on the basis of individual coping characteristics (see below). Gilts with specific coping characteristics were identified at a very young age (2-4 days) by means of a backtest. It was previously shown that for pigs with extreme low or high resistance in the backtest, relationships exist between responses in this test and behavioural and physiological ways to cope with environmental changes at a much later age (Hessing et al., 1994b; Ruis et al., 2000). Extreme low and high resisting piglets in the backtest are considered to represent reactive and proactive animals,

respectively (Hessing et al., 1994b; Ruis et al., 2000). It was shown, for instance, that high resistant pigs were the more aggressive animals in group-feeding competition tests at 10 and 25 weeks of age (Ruis et al., 2000). Low-resistant pigs, on the other hand, had a higher hypothalamic activation to a novel environment test (at 10 weeks of age), to routine weighing (at 25 weeks of age), and to administration of a high dose of ACTH (at 24 weeks of age) (Ruis et al., 2000). Low-resistant pigs in the backtest were later also found to more inhibited to approach a novel object (at 3 and 8 weeks of age (Hessing et al., 1994b)) and to enter a novel surrounding (at 10 weeks of age (Ruis et al., 2000)), leading to longer latencies (to contact).

In the present study, gilts with specific coping characteristics were socially (physically and visually) isolated by removal from their litter-mates at seven weeks of age. Endocrine, behavioural and immunological effects were subsequently studied during 3 weeks. Moreover, production in terms of body growth and feed-efficiency (gain/feed) was examined. To assess their emotional state after one week of isolation, gilts were placed into a novel environment and exposed to a novel object. Stress responses to these novel stimuli are often associated with emotions like fear or excitability (Andersen et al., 2000b; Boissy and Bouissou, 1995; Hopster et al., 1999; Von Borell and Ladewig, 1992). After five weeks of isolation, post-mortem observations were done to determine stomach wall ulceration and weights of adrenals and thymus.

Materials and Methods

All procedures in this study were conform the requirements of the Animal Care and Use Committee of the Institute for Animal Science and Health in Lelystad (ID-Lelystad), The Netherlands. Figure 1 shows the timing of management and experimental procedures.

Selection of reactive and proactive gilts

The study was done in three identical and consecutive trials (batches) from January to July. Crossbred gilts (Great Yorkshire x (Great Yorkshire x Dutch Landrace)) from the Experimental Farm for Pig Husbandry at Raalte in The Netherlands were used. They were born in farrowing pens (3.60 x 2.20 m) with partly (50%) slatted concrete floors. Within 1 day after birth, piglets were weighed and received an ear tattoo for identification. Prior to further routine procedures, piglets were subjected to a backtest (manual restraint) between 2-4 days of age, by the procedure described by Ruis et al. (2000). Briefly, in this test, a piglet is put on

its back during 1 min and the number of escape attempts (behavioural resistance) is used to characterize the animal.

Experimental procedures		**Routine procedures**	
b	backtest	**w**	weighing
s	start observations/samplings	**e**	eye-teeth and tail clipping
i	isolation	**we**	weaning
ne	novel environment	**t**	transport
no	novel object	**r**	relocation
p	post-mortem observations		

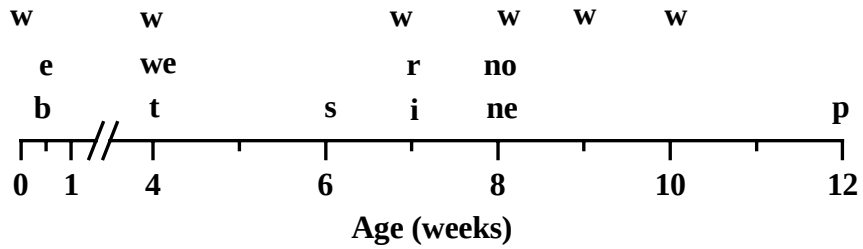

Figure 1. Timing of experimental and routine procedures.

Extreme responders, i.e. the low resistant (LR; two or less escape attempts) and high resistant (HR; five or more escape attempts) were selected, representing the reactive and proactive gilts, respectively (Ruis et al., 2000). A total of 281 female piglets were tested, of which 74 animals (roughly the bottom 25% of the distribution) were classed as LR and 70 animals (roughly the top 25% of the distribution) as HR. The population distribution and the selection criteria were similar to that reported before by Ruis et al. (2000) (see also Figure 2). Selected piglets remained in their litters until weaning (at 4 weeks of age). Shortly after weaning, selected gilts were transported to an experimental farm in Lelystad, The Netherlands, which is part of the Institute for Animal Science and Health (ID-Lelystad), where the actual experiment took place. Litter-mates (3 to 5 animals in 38 litters) were kept together and were not mixed with animals of other litters. These litters, which lack 'medium' responders, were standarized as much as possible according to pen-mates. In litters of 3 and 4 gilts, at least 1 LR and 1 HR gilt was present, and litters of 5 gilts consisted of at least 2 LR and 2 HR gilts.

Isolation procedure and management

In each trial, experimental testings took place in three adjacent rooms. Groups of litter-mates were randomly allocated to these rooms, in which temperature (kept between 19 and 21^{0}C) and lighting (lights on from 06.00 to 18.00 h; total lux varying from 50 to 100) were controlled. Pen-size was 2.35 x 1.70 m and the concrete floors were part-slatted. Food (commercial pelleted dry diets) and water (from nipple drinkers) were available ad libitum. During 2 weeks, pigs were kept in this environment without experimental intervention, but with habituation to housing and human presence. For the isolation procedures, starting at the age of 7 weeks, 12 LR and 12 HR gilts were removed from their litters, and housed individually in 1.80 x 0.85 m pens on partly slatted floors.

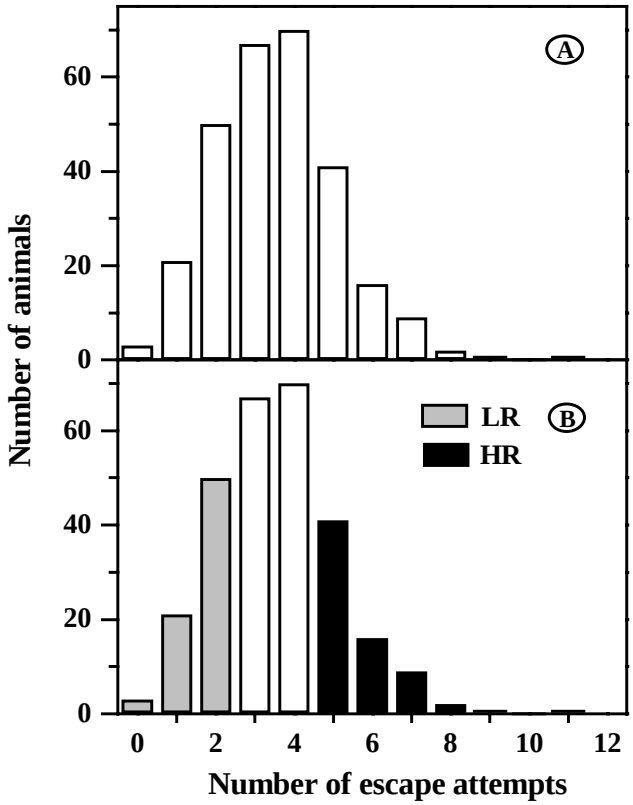

Figure 2. (A) The histogram of escape behaviour (number of escape attempts) of gilts in a 60-s backtest performed at 2-4 days of age (n = 281); (B) The same distribution as above, but after classification of extreme responding gilts as either low resistant (LR; two or less escape attempts; n = 70) or high resistant (HR; five or more escape attempts; n = 74) (see also Ruis et al., 2000).

To minimize litter-effects, gilts were chosen from as many as possible litters (maximally 2 gilts from 1 litter: 12 LR gilts from 11 litters and 12 HR gilts from 10 litters), with initial weight being balanced across the two experimental groups. A change of room (relocation) was always part of the isolation procedure, and numbers of LR and HR were equal in each room. Within each trial, isolations occurred on four different days, with 1 LR and 1 HR gilt being housed individually on one day. During the individual housing, lasting for 3 weeks, gilts were able to hear other pigs, but they were not able to have visual and physical contact (social isolation). Regular contact (frequency and length) between caretakers and animals was maintained, and should not have confounded with the outcome of the experiment. Isolation always started in the morning between 8.00 and 10.30 h. Gilts which were not isolated, were allocated to mixing procedures, described elsewhere (Ruis et al., submitted: Chapter 7).

Sampling procedures for hormonal and immunological measurements

Blood, saliva and urine samplings and processings took place according to procedures described by Ruis et al. (submitted: Chapter 7). Blood samples were collected 2 days prior to, and after 1 and 3 weeks of isolation (between 9.00 and 11.00h). Blood was obtained by puncturing the jugular vein. The duration of handling and sampling took approximately 1 min per pig, and should not have confounded with measurements of baseline cortisol. Before isolation, however, in some cases 2 gilts of the same group were sampled (see previous section). In these few cases, order of samplings were randomized, and blood sampling of one gilt may have affected the hormone levels measured in the other pig. The greater portions (8 ml) of the blood samples were transferred to ice-cold EDTA coated tubes and centrifuged (at 4^0C, 10 min, 2000 *g*) within 30 min. Then, 1.5 ml aliquots of plasma were either frozen at -20^0C (for cortisol measurements) or at -80^0C (for ACTH and prolactin determinations). Two ml blood samples originating from samplings at the above timepoints, were transferred to heparin coated tubes and kept at room temperature. They were assayed for leucocyte counts within a few hours.

Saliva samples were taken by allowing animals to chew on cotton buds, according to a procedure described by Ruis et al. (1997). Samples were taken 15 min before, and 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min after the start of isolation. Some of these samplings (at 15, 30 and 45 min) coincided with behavioural samplings. At these timepoints, behavioural observations were interrupted, leading to 1 or 2 missing samples. Further saliva sampling was done on

days -2, 1, 2, 7, 14 and 21, when a single sample was taken between 8.00 and 10.00 h. These samplings did not interfere with behavioural observations. Finally, saliva was gathered in the novelty test (see further). Saliva was stored at -20^0C until analysis for cortisol.

Urine samples were collected in early morning periods (between 6.00 and 8.00 h). Collections, by awaiting spontaneous voidings (Ruis et al., submitted: Chapter 7), took place 2 days before, and 1, 3, 7, 14 and 21 days after the start of isolation. On average, 10 gilts of each type were succesfully sampled at the different timepoints. Before storage at -20^0C (for measurements of catecholamines and creatinine) samples were adjusted to pH 3 using formic acid.

Hormonal and immunological measurements

To assess hypothalamic-pituitary-adrenal (HPA) activity, concentrations of plasma ACTH, plasma cortisol and salivary cortisol were determined as previously described (Ruis et al., 2001). Plasma ACTH and salivary cortisol concentrations were measured by radioimmunoassay procedures, and plasma cortisol concentrations by means of a fluoroimmunoassay. Plasma concentrations of prolactin were quantified in one assay, by means of a radioimmunoassay (Erkens et al., 1992; Ruis et al., 2001). Urinary catecholamine (noradrenaline and adrenaline) and creatinine concentrations were determined as described elsewhere (Ruis et al., submitted: Chapter 7). Briefly, catecholamines were assayed using a high performance liquid chromatography (HPLC) procedure with electrochemical detection, following a two step extraction. Creatinine levels were determined using a colorimetric quantitative reaction (Boehringer PAP-method). Catecholamines levels were expressed as ratios to creatinine concentrations: noradrenaline/creatinine (NC) and adrenaline/creatinine (AC) ratios. Immunological characteristics were determined by measures of percentages of lymphocytes and neutrophils (Ruis et al., 2001). For this purpose, a total of 100 cells was counted microscopically, in which these leucocytes were differentiated.

Body temperature

To estimate body temperature, a thermometer was used which was inserted in the ear (ThermoScan®, IRT 3020, Braun, Germany). As a validation of this type of thermometry, comparisons were made with rectal temperatures (Ruis et al., submitted: Chapter 7). Within 10 s, temperature was measured twice, and the average value was used for analysis. Temperatures were measured twice before isolation (at -7 days and -15 min), and 1, 3 and 5 h, and 1, 2, 7, 14 and 21 days

after the start of isolation. Except for those on the day of isolation, measurements were always done between 9.00 and 11.00 h, and did not overlap with the collection of behavioural data. Temperature measurements at 3 and 5 hour after the start of isolation were done just before behavioural observations in the home pen (see next section).

Home pen behaviour

Gilts were observed in their home pens during specific 30 min periods, in which the behaviour of each animal was scan sampled at 1 min intervals (a total of 31 observations for each 30-min period). The ethogram of recorded behaviours is listed in Table 1. On the day of isolation, observation periods were started from time 0 of isolation and then at 30 min, 3 and 5 hours after the start of isolation. Additionally, behaviour was observed on 2 days prior to isolation, and on 1, 2, 7, 14 and 21 days after the start of isolation. On each of these observation days, behaviour was scan sampled at 1 min intervals during a single 30-min period (always between 8.00 and 10.00 h). Behavioural data were expressed in percentages of all (total) behavioural observations (except for vocalizing, which could coincide with other behaviours).

Behavioural, cortisol and cardiac responses to novelty

After 1 week of isolation, each gilt was subjected to a novelty test consisting of two novel stimuli, according to procedures described by Ruis et al. (2001). The order of testing of individual gilts was randomized. Handling and transport before the test was standardized as much as possible. After removal from their home pens, individual gilts were gently driven into a startbox (through a corridor for 10-20 m). Pigs were introduced into a novel arena (3.8 x 3.0 m) following opening of the startbox (novel environment: NE). A pig was left in the novel arena for a total of 15 min during which its behaviour was recorded via a video camera. Latency to leave the startbox and locomotion were analysed afterwards (Ethovision®, Noldus Information Technology, Wageningen, The Netherlands). Number of vocalizations were recorded directly throughout testing. Ten min after opening the startbox, a novel object (a yellow and a grey bucket tied together) was lowered from the ceiling onto the floor and then lifted to approximately 0.5 m above the floor. Behavioural parameters used in this novel object test (NO) were: contact latency, number of contacts, total time of contact, and number of vocalizations. To determine the cortisol response to the novelty test (NE and NO), saliva was sampled 5 min before, and 5 and 15 min after testing.

Table 1. Ethogram of the behavioural measures

Behaviour		Definition
Exploring		Rooting, sniffing, touching the pen
Defecation/ urination		Self-explanatory
Inactive	Sleeping	Lying with eyes closed
	Lying	Lying with eyes open
	Sitting	Standing on fore-legs, hind quarter on the floor
	Standing	Standing inactive, may be between activities
Ingestive	Feeding	Time spent with head in the feeder and chewing feed
	Drinking	Use of water nipple to obtain water
Vocalizing		Total vocalizations: grunts and squeals
Walking		Walking or running through the pen

Two min before allowing gilts to enter the novel arena, i.e. immediately after being driven into the startbox, they were equipped with a commercial heart rate monitor (Vantage® NV, Polar Electro Oy, Kempele, Finland). This monitor allowed to measure heart rate and heart rate variability (HRV) in the time domain. The following indices of cardiac activity were determined (Sgoifo et al., 1999): (1) mean HR (beats per minute: bpm), as measured from the time between two successive R-peaks of the ECG (R-R intervals: RR, ms); (2) overall HRV (sympathetic- parasympathetic autonomic balance), as estimated by (a) the standard deviation of the mean RR (SD, ms), and (b) the ratio between the standard deviation of the mean RR and the mean RR (SD/RR, coefficient of variance); and (3) root mean square of successive RR differences (r-MSSD, ms), which estimates the parasympathetic influence on HRV. To gain knowledge on cardiac reactivity prior to isolation, heart rate and HRV were determined in the home pen during 9-min periods. This was done between 3 to 5 days before isolation. Because at this time gilts were still housed in groups, heart rate monitors were protected from damage by fastening them under a belt made of elastic band. This procedure caused some disturbance, and accordingly may have had the potential to lead to (mild) stress (Ruis et al., 2001).

Production

Shortly before the start of isolation, and once a week during 3 weeks thereafter, all pigs were weighed. Feed intake was determined by keeping a daily record of all feed added to, and the weight of, the feed hoppers. Feed intake, live-weight gain, and gain/feed ratio were calculated per week.

Post-mortem examinations

Five weeks after the start of isolation, pigs of trials 1 and 3 were sacrificed for examinations of pathological changes in the pars oesophagea of the stomach, weights of adrenal glands and thymus, and permeability of gut epithelium. The appearance of the pars oesopagea of the stomach was scored for any development of hyperkeratosis and ulceration. A scoring protocol ranging from 0 to 5 was used (Hessing et al., 1994a). Adrenal glands and thymus were weighed and these weights were expressed relative to body weights. Methodology and results of gut permeability will be described elsewhere (Van Kalkeren et al., submitted).

Statistical analysis

Data was analyzed with an analysis of variance model with main effects for type (LR or HR) and trial (1-3). For analysis of percentages a logistic regression model was employed with a multiplicative overdispersion factor. Counts were analyzed as overdispersed Poisson data on a logarithmic scale. Latency times were also analyzed on a logarithmic scale. Due to the low incidence of stomach ulceration, only descriptive statistics are given for this variable (percentages, numbers). Hormonal and temperature changes within animals were analyzed with a paired t-test. All calculations were performed with the statistical programming package Genstat 5® (1993). Differences were considered significant if $p<0.05$. Data are presented as mean±SEM.

Results

Hormones and immunology

Salivary cortisol concentrations increased significantly ($p<0.01$) after isolation in both types of gilts. However, during the first 30 min, the increase was higher in LR gilts than in HR gilts (Figure 3). LR and HR gilts did not differ in salivary cortisol values following the initial 30-min period, and concentrations returned to pre-isolation values within 3 hours. At 1 and 3 weeks of isolation, (changes in) plasma ACTH, cortisol and prolactin concentrations did not differ between LR and HR gilts (see also Table 2). However, when compared to values

before isolation, isolation caused significant (p<0.05) changes in percentages of lymphocytes and neutrophils in HR gilts at 3 weeks after the start of isolation (changes in %: -5.58±3.18 and 5.33±3.37, respectively), and not in LR gilts (changes in %: 1.75±3.17 and -1.33±3.37%).

Following isolation, changes in urinary NC ratios differed significantly (p<0.05) between LR and HR gilts, with the NC ratio being more elevated in LR pigs at 1 week of isolation (Figure 4). After 3 weeks of isolation, the NC ratio tended (p=0.06) to be higher in HR than in LR gilts. No significant differences in (changes in) AC ratios between LR and HR gilts were found.

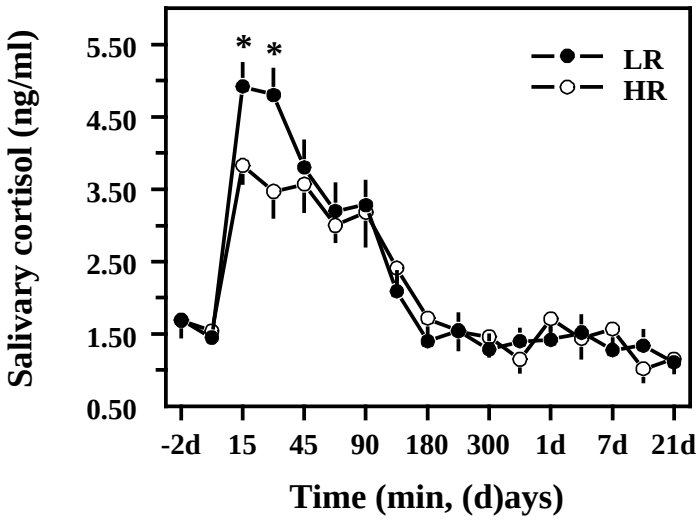

Figure 3. Mean(±SEM) salivary cortisol concentrations of LR (n = 12) and HR (n = 12) gilts during 3 weeks of social isolation. *Significant difference (p<0.05) between LR and HR gilts. For significant changes within LR and HR gilts, and significant differences in changes between LR and HR gilts, see Results.

Body temperature

Body temperature decreased significantly (p<0.05) in response to isolation in both LR and HR gilts (Figure 5). After 3 weeks of isolation, body temperatures in HR gilts were still lowered, while in LR gilts body temperatures did not differ from pre-isolation values beyond the first day of isolation. At day 7 of isolation, the difference between the two types of gilts was significant (p<0.05).

Table 2. Plasma hormone concentrations and percentages of circulating leucocyte subsets (mean±SEM) for LR (n = 12) and HR (n = 12) gilts during 3 weeks of social isolation.

		Time relative to the start of social isolation		
Variable	Type	-2 days	1 week	3 weeks
ACTH (pg/ml)	LR	59.0±29.5	62.7±10.4	48.4±18.8
	HR	102±30.9	49.7±10.3	61.0±18.8
Cortisol (ng/ml)	LR	30.1±3.7	31.4±3.3	30.0±3.7
	HR	29.5±3.9	30.3±3.4	28.0±3.7
Prolactin (ng/ml)	LR	1.20±0.16	1.30±0.24	1.42±0.23
	HR	1.52±0.18	1.58±0.25	1.68±0.22
Lymphocytes (%)[1]	LR	58.41±3.16	57.08±2.91	60.16±3.50
	HR	63.62±3.22	62.25±2.98	58.04±3.61
Neutrophils (%)[1]	LR	39.42±3.04	41.60±2.91	38.09±2.79
	HR	35.42±3.07	36.92±3.02	40.75±2.78

[1]Significant (p<0.05) difference between LR and HR gilts in changes in % leucocyte subsets: values at 3 weeks compared with those at -2 days (see also Results section).

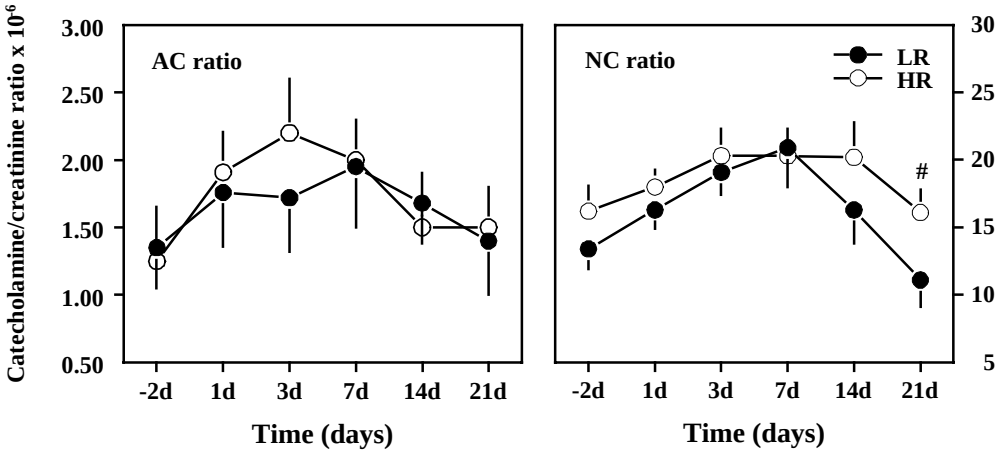

Figure 4. Mean(±SEM) urinary catecholamine concentrations of LR (average sample size: n = 10) and HR (average sample size: n = 10) gilts during 3 weeks of social isolation. #Tendency for a difference (p=0.06) between LR and HR gilts. For significant changes within LR and HR gilts, and significant differences in changes between LR and HR gilts, see Results.

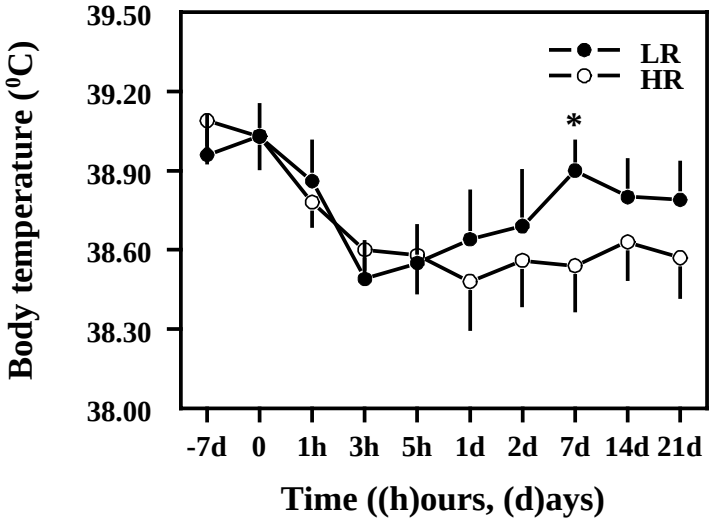

Figure 5. Mean(±SEM) body temperatures of LR (n = 12) and HR (n = 12) gilts during 3 weeks of social isolation. *Significant difference (p<0.05) between LR and HR gilts. For significant changes within LR and HR gilts, and significant differences in changes between LR and HR gilts, see Results.

Behaviour in the home pen

Before isolation, patterns of different behaviour did not differ between LR and HR gilts (Figure 6). Isolation caused a significant (p<0.01) increase in exploratory behaviour. The two types did not differ in this behaviour on the first day of isolation, as observed for specific 30-min periods and for pooled 30-min periods on the first day of isolation. However, thereafter, LR gilts were generally more often observed to explore than HR gilts (% exploration for pooled 30-min periods beyond the first day of isolation: 20.3±2.6 vs. 12.7±1.8; p<0.05). With regard to specific 30-min periods, a significant difference in exploration was observed at 1 day of isolation. Initially, HR gilts walked more than LR gilts, but this difference disappeared after 1 hour of isolation. At 1 day of isolation, HR gilts showed a higher level of behavioural inactivity, while no differences were observed at the other timepoints. Vocalizing was significantly increased in response to isolation, being elevated during the entire 3-week observation period (p<0.05 at least) for both types of gilts. Characteristically, HR gilts vocalized more than LR gilts, which was demonstrated for pooled 30-min periods on the first day of isolation (% vocalizing: 39.3±3.1 vs. 31.2±3.3; p<0.05), for pooled 30-min periods beyond the first day of isolation (% vocalizing: 28.4±3.1 vs. 18.1±3.7; p<0.05), but not for specific 30-min periods. Feed and water intake (ingestive behaviour) did not differ between both types of gilts. Finally, LR gilts were

generally more often observed to defecate/urinate compared to HR gilts (on the first day of isolation: 0.78±0.2 vs. 0.21±0.15%, pooled 30-min periods: $p<0.05$; beyond the first day of isolation: 1.24±0.3 vs. 0.48±0.18%, pooled 30-min periods: $p<0.05$). However, this behaviour represented only a very small percentage of total behavioural observations.

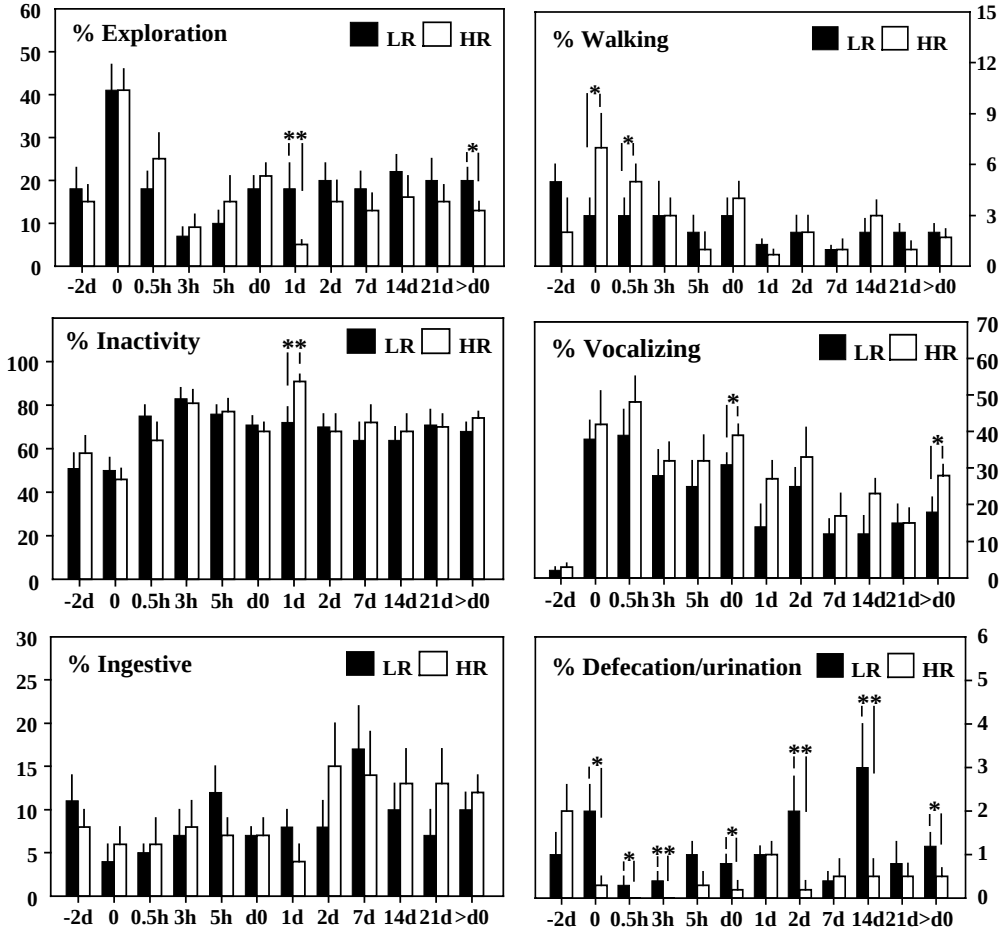

Figure 6. Behaviour of LR (n = 12) and HR (n = 12) gilts during 3 weeks of social isolation. Gilts were observed in their home pens during specific 30-min intervals (timepoints: **h**ours, **d**ays). Pooled 30-min periods on the first day of isolation: d0. Pooled 30-min periods beyond the first day of isolation: >d0. Behavioural elements were expressed in percentage (mean±SEM) of total behaviours. Significant differences within timepoints between LR and HR gilts: $^{**}p<0.01$, $^{*}p<0.05$. For significant changes within LR and HR gilts, and significant differences in changes between LR and HR gilts, including pooled observations, see Results.

Behavioural, cortisol and cardiac responses to novelty

Table 3 shows the behavioural and cortisol responses to the novel environment (NE) and the novel object (NO). With respect to behavioural observations in these novelty tests, the only significant difference between LR and HR gilts was noticed in the NO, in which HR gilts vocalized more often. The salivary cortisol response to overall testing was higher in LR gilts compared to HR gilts. With regard to cardiac activities, overall heart rate variability (SD/RR; during the NE) and parasympathetic activity (r-MSSD; during the NE and NO) were higher in HR gilts (Table 4). Heart rates did not differ between the two types of gilts. Before isolation, LR and HR gilts did not differ in heart rate variability and average heart rate, as observed in their home pens. Parasympathetic activity (r-MSSD), however, tended to be higher in LR gilts before isolation ($p<0.1$; Table 4).

Table 3. Behavioural and cortisol responses (mean±SEM) of LR and HR gilts to the novelty test at 1 week of isolation.

		Type	
Variable		LR (n = 12)	HR (n = 12)
NE	Latency to enter (s)	24.2±6.6	24.3±6.7
	Locomotion (m)	100±7.3	101±7.7
	Vocalizations (number)	124±22	137±21
NO	Contact latency (s)	21.3±8.0	23.9±7.9
	Number of contacts	10.8±1.6	12.6±1.6
	Contact time (s)	37.4±7.2	47.3±7.2
	Vocalizations (number)[1]	71±10	113±10
NE+NO	Cortisol response (ng/ml)[2]	2.54±0.31	1.60±0.35

NE (novel environment; 10-min period); NO (novel object; 5-min period). Significant difference between LR and HR gilts: [1]$p<0.01$, [2]$p<0.05$.

Production

LR and HR gilts did not differ significantly in body weight gain and feed intake (Table 5). However, in the second week of isolation, the gain/feed ratio was significantly ($p<0.01$) lower in LR gilts compared to HR animals. This effect of coping characteristics on gain/feed was not observed in the other weeks, nor when averaged over the whole 3-week isolation period.

Table 4. Heart rate and heart rate variability (mean±SEM) of LR (n = 12) and HR (n = 12) gilts, before isolation, and during the novelty test at 1 week of isolation.

		Test		
Variable	Type	before isolation	NE	NO
HR (bpm)	LR	173±4.9	165±4.6	163±5.0
	HR	178±5.1	169±4.7	162±4.7
SD (ms)	LR	24.2±2.6	34.0±5.0	38.8±5.3
	HR	21.4±2.7	42.7±5.1	39.1±5.1
SD/RR	LR	0.07±0.01	0.07±0.01[1]	0.10±0.01
	HR	0.06±0.01	0.11±0.01	0.10±0.01
r-MSSD (ms)	LR	0.35±0.04[2]	0.39±0.13[1]	0.58±0.14[1]
	HR	0.23±0.05	0.81±0.14	0.97±0.14

Heart rate (HR), standard deviation (SD), coefficient of variance (SD/RR) and root mean square of successive RR differences (r-MSSD), before isolation (in the home pen: 9-min period), during the NE (novel environment; 10-min period) and during the NO (novel object; 5-min period). Significant difference between LR and HR gilts: [1]$p<0.05$, [2]$p<0.1$ (tendency).

Table 5. Production characteristics (mean±SEM) of LR and HR gilts during 3 weeks of social isolation.

		Type	
Variable	Period	LR (n = 12)	HR (n = 12)
Feed intake, kg	Week 1	8.99±0.52	8.32±0.50
	Week 2	10.0±0.65	9.35±0.64
	Week 3	10.75±0.69	11.08±0.68
	Total period	*29.74±2.04*	*28.75±2.01*
Weight gain, kg	Week 1	7.47±0.47	6.93±0.47
	Week 2	5.74±0.42	6.03±0.40
	Week 3	6.18±0.38	6.86±0.38
	Total period	*19.39±1.19*	*19.83±1.19*
Gain/feed, kg/kg	Week 1	0.83±0.01	0.83±0.02
	Week 2[1]	0.57±0.02	0.64±0.02
	Week 3	0.59±0.04	0.63±0.04
	Total period	*0.66±0.02*	*0.70±0.02*

[1]Significant ($p<0.01$) difference between LR and HR gilts.

Post-mortem observations

Most isolated gilts had intact (69%; score of 0) or slightly damaged (25%; hyperkeratosis and no ulceration; scores of 1 or 2) stomach walls. The prevalence of more severe (score of 3 or more) stomach wall damage was only determined for one HR gilt, which showed severe hyperkeratosis and ulceration (score of 4) of the pars oesophagea. Weights of adrenals (in mg/kg: 66±2.7 and 68±4.1, respectively) and thymus (in g/kg: 2.76±0.11 and 3.02±0.27, respectively) did not differ between LR and HR gilts.

Discussion

Our results show that social isolation was perceived as a stressful condition by both types of gilts. This was indicated by physiological changes which were considered indicative for a higher state of stress, such as an acute release of cortisol (Harbuz and Lightman, 1992, Minton, 1994) and a (less) acute decrease in body temperature (De Jong et al., 1998; Chen and Herbert, 1995). Consistent with earlier findings, social isolation also induced behavioural changes like an increase in exploration (Carbonaro et al., 1992; Jensen et al., 1997; Sahakian et al., 1977) and more vocalizing (Boissy and Bouissou, 1995; Rushen et al., 1999b; Von Borell and Ladewig, 1992). Whereas exploration may represent a search for social contact (social motivation), vocalizing may be guided by both social motivation and fear (Ruis et al., 2001).

Stress responses to isolation, however, differed in magnitude and/or duration between LR and HR gilts. The acute increase in salivary cortisol, for instance, was higher in LR gilts as compared to HR gilts. The same comparison showed that LR animals were generally more explorative, as shown for pooled observations beyond the first day of isolation. HR gilts, on the other hand, were more ‘restless’ than LR gilts, which was especially seen shortly after isolation. HR gilts performed more walking/running behaviour during the first hour, and showed a higher level of vocalizing (pooled observations) during the first day of isolation. In the longer term, HR gilts vocalized on average more than LR gilts did (pooled observations beyond the first day of isolation; Figure 6). Moreover, after 3 weeks of isolation, the urinary noradrenaline/creatinine (NC) ratio was higher in HR gilts. This difference could not be explained by a difference in behavioural activity (Fibiger et al., 1984). These characteristics of LR and HR gilts agree with expected reaction patterns on the basis of preferred ways of coping. Reactive copers, here represented by the LR gilts, were previously shown to have a relatively high HPA axis reactivity and a high explorative motivation under challenging conditions (De

Boer et al., 1990; Koolhaas et al., 1999; Ruis et al., 2000). Responses of HR gilts, on the other hand, were more characterized by proactivity. Proactive rodents were observed to be more active in response to a stressor, by actively seeking a way to remove themselves from the source of stress (Benus et al., 1991; Bohus et al., 1987; Fokkema et al., 1995; Koolhaas et al., 1999). This may resemble the higher level of 'restlessness' of HR gilts at the start of isolation. Physiologically, the higher domination by the sympathetic nervous system in HR pigs agrees with observations of proactive rodents (Benus et al., 1991; Bohus et al., 1987; Fokkema et al., 1995; Koolhaas et al., 1999) and pigs (Hessing et al., 1994b), which predominantly react with a sympathetic stress response.

A dominance of the sympathetic nervous system in HR gilts was not observed in the novelty test, during which average heart rates did not differ between the two types of gilts. Nevertheless, we argue for a higher sympathetic activity in HR gilts, but this was accompanied by an increase in parasympathetic activity. The latter was evidenced by a higher r-MSSD in HR gilts compared to LR gilts. The r-MSSD only takes the high frequency variations of RR intervals into account, which specifically quantify the influence on heart rate of the parasympathetic branch of the autonomic nervous system (Sgoifo et al., 1999). The higher heart rate variability (SD/RR) in HR gilts, observed in the novel environment, may substantiate this vagal counter regulation of sympathetic activation (Sgoifo et al., 1999). A predominant parasympathetic reactivity, however, has often been ascribed to the more reactive type of animal (Bohus et al., 1987; Fokkema et al., 1995; Koolhaas et al., 1999). Indeed, prior to isolation, during cardiac monitoring in groups of pigs, parasympathetic activity tended to be higher in LR compared to HR gilts. We therefore suggest that the relatively high parasympathetic activity in HR gilts represented a way to compensate for an increase in sympathetic tone during stress caused by novelty. A maintenance in sympathovagal balance during stress-inducing situations was reported before (De Jong et al., 1998; Ruis et al., submitted: Chapter 7). In addition, it may be argued that the novelty test was less stressful for the LR gilts, leading to a relatively small parasympathetic response. This may be substantiated by behavioural observations. On the basis of preferred coping responses to environmental challenges (Hessing et al., 1994b, Ruis et al., 2000), it may be expected that LR gilts more gradually explore novel surroundings or novel objects, leading to longer latencies to contact (Hessing et al., 1994b, Ruis et al., 2000). However, differences in latencies to leave the startbox and to contact the novel object were not observed in the present experiment. This possibly indicates that the novelty challenges were relatively

more demanding for HR gilts. In the present study, several long-term observations in the home pen support a difference in the state of stress between LR and HR gilts, as shown by differences in the temporal patterns of stress responses. In general, these differences point to a prolonged (chronically) higher general state of stress of HR gilts. Body temperature, for instance, did not recover in HR gilts within the 3-week observation period. In contrast, these values were not found to differ from pre-isolation values beyond the first day of isolation in LR gilts. Moreover, when comparing values at 3 weeks with those prior to isolation, a decrease in percentage of lymphocytes and an increase in percentage of neutrophils indicated a higher state of stress in HR gilts (McGlone et al., 1993b; Ruis et al., 2001; Stefanski and Engler, 1998). In LR gilts, no changes in percentages of these leucocyte subsets were observed. The incidence of stomach ulceration was very low, and no statistically founded conclusions can be derived. However, the only animal showing ulceration was a HR gilt, which may support the thought of a higher vulnerability of proactive animals to the formation of ulcers, when stress is uncontrollable (McCabe et al., 2000; Koolhaas et al., 1999). Our arguments for a situation of chronic stress in HR gilts, but not in LR animals, could not be supported by data on weights of adrenals and thymus. It was previously reported that chronic stress conditions are able to, respectively, enlarge and reduce the size of adrenals and thymus (Baldwin et al., 1995; Ruis et al., 1999; Selye, 1950), but we were not able to demonstrate differences between the two types in the weights of these organs.

LR gilts showed a lower gain/feed ratio in the second week of isolation and a more elevated NC ratio at 1 week of isolation. At least to some extent, a higher behavioural activity of LR gilts may have accounted for the effect on these variables, rather than being solely attributed to stress (Fibiger et al., 1984; Stookey and Gonyou, 1994). Defecation/urination behaviour was only rarely observed, and it may be questioned whether registration of this short-lasting behaviour can be done properly with scan sampling. Nevertheless, defecation/urination was slightly more often observed in LR gilts. Again, a higher behavioural activity may have played a role: higher activity in itself may lead to more time spent in the dunging area, thereby triggering defecation/urination behaviour.

To conclude, our results indicate that LR and HR gilts differed in their ways to adapt to the social isolation challenge, as seen by several differences in the temporal dynamics of stress responses. Some variables may point to a higher state of stress in LR or reactive gilts, but the general impression is that these animals recovered more quickly from the imposed social isolation than HR or proactive

gilts did. Especially for the latter animals, this social challenge seemed to become a chronic stressor. Although we cannot simply generalize between stressors, it may be hypothesized that a better adaptation of LR gilts to social isolation may represent a general better ability to adapt to a variety of challenges, occurring in intensive husbandry conditions. Conditions which are difficult to control may especially impose a risk for welfare and health of HR or proactive pigs. However, further research is needed to confirm this hypothesis.

Chapter 9

General and summarizing discussion

Integration and discussion of the findings

The main purpose of this thesis was to study (psycho)social aspects of modern pig farming and its consequences for animal welfare. By investigating acute and long-term effects of social defeat, it was aimed to find out whether well-documented reductions in welfare following social mixing may be ascribed to initial fighting for dominance status. Moreover, it was investigated whether interactions with familiar animals following a social stressor improve the ability to adapt to the stressor, and what the consequences are for welfare when no social contact at all is allowed. Finally, attempts were made to identify pigs with different coping strategies, which may explain the nature of animal interactions within social groups and differences in the ability of individual adaptation to environmental changes in general. A definition of welfare and its assessment by a multidimensional approach is given in Chapter 1. One promising and convenient parameter for measurements of the stress response, i.e. salivary cortisol, was more thoroughly investigated (Chapter 2). Its circadian rhythmicity was studied in relation to age, gender and stress. For measurements of salivary cortisol, a radioimmunoassay (RIA) was optimized and validated. One major finding was that the timing of stressor application importantly influenced the acute cortisol response: the increase was higher in morning periods. This finding was accounted for as much as possible in the other experiments (Chapters 3-8) of the project, by restricting (the start of) applications of stressors or testings to certain parts of the day. In the following, an integrated discussion on Chapters 3-8 is given, by raising the main topics and findings of this thesis.

Social defeat versus social support in rats: a model for pigs

Social defeat

One important source of social stress in pigs is the aggression that occurs when unfamiliar conspecifics are mixed, which is generally recognized as a major welfare concern in commercial pig farming. However, for practical reasons, mixing of unfamiliar pigs is an important management procedure in pig farming. In the current thesis an attempt was made to elucidate which social aspect(s) constitute(s) the highest risk for pig welfare during mixing, by separating the effects of initial fighting for dominance, from the continuing social stress that may exist through a permanent coexistence of unfamiliar animals. Fighting for dominance is initially characterized by mutual aggression, and is therefore expected to be a severe acute stressor for both the winner and the loser of the fight. However, it has been repeatedly demonstrated that the stress of being defeated leads to stronger and

more prolonged stress responses (Schuurman, 1980), which was also substantiated by our results in Chapter 7. Haller et al. (1996) suggest that dominance is associated with reductions in corticosteroids, whereas submission elicits a strong increase. The concomitant lack of control of being defeated may thus cause a threat to welfare, especially because a long-lasting effect of social defeat may be observed, characterized by long-term adverse behavioural and physiological changes (Koolhaas et al., 1997b; Miczek et al., 1990). The alterations in behaviour and physiology resemble those in depressed humans and therefore the social defeat model in rats is often used for studies into this type of human psychopathology (Koolhaas et al., 1997b).

Modulating effects of the social environment

In this project, the social defeat model in rats was used to gain more insight in potential harmful long-term effects of acute social defeat in pigs. However, one limitation of this rat model is that isolated animals are used. This is rather unnatural, because, like pigs, wild rats live in social groups. These groups consist of a number of related males (often one dominant and several younger males) and a varying number of females. The validity of the defeat model may importantly be increased when housing conditions are used as an experimental variable. This will mimic different social settings which individuals may experience in everyday life. We investigated the effect of the social environment and found striking differences between socially defeated rats that were individually housed and those that were housed together with familiar conspecifics following the acute social stress. Compared to group-housed rats, individually housed animals showed reduced body growth, high sensitivity to repeated stress, low open field activity, high anxiety and hyperactivity of the HPA-axis. These characteristics of defeated and isolated rats more or less agree with indicators of depression in humans, such as decreased general activity, loss of weight and abnormal high HPA-axis (re)activity. Whether a depression-like state may develop in defeated rats thus strongly depends on contextual social factors. The presence of familiar conspecifics importantly reduces the long-term negative effects of social defeat. Such an amelioration of stress responses by the social environment is called *social support (social buffering)*. Social support cannot be provided by any conspecific, but only by bonding partners (Sachser et al., 1998). The groups of familiar rats used in Chapter 3 consisted of litter-mates and accordingly social bonding between group-members was expected to be strong. The phenomenon of social support is probably widespread among mammals. As McGlone (1990) mentioned: 'It seems that misery loves company,

even in the animal kingdom'. By maintaining the availability and accessibility of bonding partners, an animal's way of living is economized and its chances of survival are increased (Sachser et al., 1998).

The importance of a (stable) social environment for pigs

Social defeat and social support

Based on the information on social defeat and social support in rats (Chapter 3), acute and long-term effects of social defeat and modulating effects of the social environment were investigated in pairs of growing gilts (Chapter 4). As for rats, it was demonstrated that social defeat constitutes a severe stressor for pigs, by causing pronounced increases in HPA-axis and sympathetic-adrenal medullary activities, and shifts in leucocyte subsets. When social defeat was followed by the possibility to interact with a familiar conspecific, i.e. a litter-mate, stress responses were generally shorter-lived and habituation to a repeated novel environment test was facilitated. This suggests that litter-mates can act as bonding partners. The increased ability of a defeated pig to adapt to the social threat through social support is considered to be positive for its welfare.

Welfare problems of mixing

Social defeat in pigs always seemed to leave some traces in the longer term, as observed by a higher sensitivity of defeated pigs to (mild) changes in their environment (Chapter 4). However, body growth of defeated pigs was not affected, which contrasts findings in pens of mixed pigs in which subordinate pigs show a depressed growth (Albinsson and Andersson, 1980; Giroux et al., 2000). In line with this, it is shown in Chapter 7 that within pairs of unfamiliar gilts body growth is lower in subordinates compared to dominants. It is therefore likely that the negative effects of the social stress of initial fighting for dominance status, and the social stress that persists through permanent coexistence of unfamiliar animals, together lead to the often observed reductions in welfare following mixing. Being a subordinate in a newly mixed group of strangers may consequently be more stressful than being a subordinate in a group of familiar pen-mates. Chapter 7 also showed that detrimental effects of mixing on body growth develop independent of social stability (stability of relations among animals in social groups). Thus, even when aggressive physical contact is low, social stress may persist through visual exposure to opponents. The higher vulnerability to environmental stimuli after social defeat, together with a reduced body weight gain when being permanently exposed to dominants, may suggest a depressive-like state in subordinate pigs.

In Chapter 5 it was studied what impact mixing of unfamiliar pigs has on specific long-term immune responses and protection against infection after vaccination with pseudorabies virus (PRV). As shown for some immune parameters (e.g. lymphocyte proliferation and IFN-gamma responses after vaccination, IgG1/IgG2-ratio after challenge) dominance in itself seemed to guarantee a higher immune response. However, when other parameters (e.g. IFN-gamma/IL-10 ratio after challenge) were considered, the immune response seemed to be more suppressed in mixed dominants than in mixed subordinates. The latter may be unexpected, also on the basis of our findings in Chapter 7, in which we argue for a more serious effect of mixing for subordinates than for dominants. However, our findings with regard to immune capacity may emphasize that the social situation imposed by mixing may not only compromise welfare of subordinates, but is also a 'uncomfortable' situation for dominants animals. The latter animals should maintain their high social status and accordingly may experience a continuous threat to control. More or less surprisingly, we also observed a gender-related difference in immune capacity (Chapter 5). It was shown that mixed barrows suffered more from immunosuppression than mixed gilts, although levels of agonistic interactions did not differ. We do not have a sound explanation, but the phenomenon may not be completely new. For example, a large scale research with 18000 pigs showed that barrows more often suffered from chronic inflammations than gilts (De Kruijf and Welling, 1988). It may be speculated that this is due to a higher HPA-activity in barrows, as being argued for in Chapters 2 and 5, leading to a lower immune capacity of males. The stress and the change in hormonal patterns which accompany castration may be the mediators of an increased HPA-(re)activity in barrows.

Individual housing

It was mentioned that the social environment not only has negative effects on individuals but can also act in a positive way. Our findings in Chapter 4 also emphasize that being able to interact with any conspecific, i.e. being socially housed, is a prerequisite to safeguard welfare of pigs. Social isolation by individual housing gradually leads to a state of reduced welfare, as shown by increases in emotional arousal and decreases in abilities to habituate to repeated 'novel' stimuli. Moving to another pen may strengthen these effects of isolation, by additionally inducing high acute stress responses to this procedure (Chapter 8). Although individual pigs may not be equally vulnerable to isolation stress (Chapter 8), it is argued that the generally higher emotional arousal of isolated pigs represents a

higher state of fearfulness (Chapter 4). Despite the indications for a disturbance of mood, body growth of isolated pigs was not negatively influenced. Several other studies have even shown a higher growth performance of individually penned animals than those kept in groups (Gomez et al., 2000; Morgan et al., 1999). We argue that body growth is confounded with direct effects of the social environment. Individually housed pigs spent more time in feeding behaviour than animals housed in groups, as a substitute for social behaviour (Morgan et al., 1999). Pigs in groups also tend to synchronize their feeding behaviour, which may lead to a competition for food, especially in case of insufficient trough space. Accordingly, food intake of (certain) members of a group may be less than desired. Despite the opportunity to feed at other times when the trough is vacant, pigs prefer to remain with group mates (Morgan et al., 1999). Thus, (normal) body growth should be carefully interpreted, and we doubt that this parameter may be used as a welfare indicator when different social settings are compared.

Coping strategies in pigs and their consequences for welfare

The present project provides further evidence of the large physiological and behavioural variation among pigs living under farm conditions, despite domestication. Importantly, at least for part of the pig population, this variation seems to be determined by fundamental differences in ways of coping, although we were not able to demonstrate the existence of specific categories of pigs (Chapter 6). Nevertheless, extremes within the population differ generally in the same behavioural and physiological parameters and to the same degree, and fulfil the criteria for (more) reactive and (more) proactive coping strategies.

Validity of the backtest to predict coping characteristics

Previously, Hessing (1994) suggested that on the basis of behavioural resistance in a backtest, pigs could be divided into reactive and passive copers. His suggested bimodality of coping responses in pigs, however, could not be replicated by others (Forkman et al., 1995), leading to confusion and debate among researchers. From our experiences with the backtest, we conclude that the distribution of reactions to this test is unimodal, with no indication for a limited number of isolated classes. However, we hypothesize that such a distribution may not exclude that animals towards the ends of the scale may have a prevalence for one or another strategy. Indeed, without wanting to create an impression that there are real categories of individuals, extreme responders to the backtest differed in levels of aggression at a later age and had different patterns of stress responses in

various situations. Low resisting pigs are generally low-aggressive and adopt a more reactive way of coping, whereas the high resistant pigs are more aggressive and represent the more proactive copers. Table 1 summarizes the behavioural and physiological characteristics of gilts which represent the (more) reactive and (more) proactive pigs according to behavioural resistance in the backtest (integrated results of Chapter 6, 7 and 8).

Table 1. Summary of the behavioural and physiological characteristics of high- and low-resistant pigs in the backtest.

	High-resistant 'proactive'	Low-resistant 'reactive'
Behaviour		
Aggression	High	Low
Cue dependency	Low	High
Escape behaviour	High	Low
Exploration	Low	High
Vocalizing	High	Low
Physiology		
HPA-axis reactivity	Low	High
Parasympathetic activity	Low	High

In Chapter 6 we argue that the quality of a backtest at a very young age is attributed to testing of rather naive animals, unaffected by previous experiences. At this age, pigs already have a clear personality, which is predominantly determined by genetic factors. In the following, we hypothesize from a motivational and functional point of view why the backtest is a valuable tool for predictions of aggression and coping responses. In the pairs of pigs which were studied in Chapter 7, proactive pigs had an equal chance to become dominant or subordinate. When proactive animals became dominant, they always behaved aggressively towards opponents. When they became subordinate, only forced submission through acts of hostility and aggression of opponents evoked aggression. Reactive animals, on the other hand, only used aggression when challenged and their aggressive intentions were much suppressed when their social positions became clear. Being restrained in a supine position during a backtest may represent forced submission, by its resemblance with certain aspects of social fighting. We therefore

argue that reactive animals more readily accept their forced subordinate status and resist less fiercely, whereas proactive animals become frustrated by bodily forced submisssion and are more eager to resist. A comparable test as the backtest is used in dogs (puppy-selection test), as a tool to predict to what extent these animals accept leadership from humans (Campbell, 1975), although this test is not generally recognized to have a predictive value for this purpose (Beaudet et al., 1994). In contrast to what Hessing et al. (1993) claimed, it is thus more likely that the backtest done with piglets represents a social than a non-social test.

Coping characteristics, aggressive behaviour and social stress after mixing

As shown in Chapter 7, proactive pigs are particularly aggressive when being dominant or frustrated. Reactive animals, in contrast, are only aggressive when challenged, i.e. when fighting for dominance, and their aggressiveness is much suppressed when social relationships are settled. The presence of reactive animals in a group may thus be of advantage for the stability of (relations between individuals in) a social group. We also showed that the most stable social relationships occurred between a reactive and a proactive pig. This may indicate that in social organizations, animals with different or complementary characteristics are needed to establish social stability and to increase the 'survival of the group'. Indeed, Hessing et al. (1994a) previously showed that group performance of pigs was best in groups with a large variation in individual aggressiveness. However, we should emphasize that when a proactive pig takes the dominant position this may not be beneficial for the welfare of a reactive subordinate. Nevertheless, most detrimental for welfare was the situation of cohabitation of two proactive pigs, characterized by relatively high levels of aggressiveness, stress and fear in both animals. The level of aggression following mixing is thus related to the coping strategy of individual pigs, but also depends on the social position of respective individuals within the social group.

Coping strategies, social isolation and effects of domestication

As described in Chapter 8, pigs with reactive and proactive features differed in their strategies to deal with social isolation, as demonstrated by several differences in the temporal dynamics of stress responses. The general impression is that reactive pigs recovered more quickly from the imposed social isolation than proactive animals. Studies with rodents showed that reactive animals more easily adapt to variable conditions and are more flexible than proactive animals. The latter animals deal better with stable environmental conditions (Benus et al., 1991;

Koolhaas et al., 1999). When this is generalized, the better adaptation to social isolation of reactive pigs may represent a generally better ability of these pigs to adapt to a variety of challenges. If this assumption is right, this may support the theory that domestication may favour a certain type of animal (Hopster, 1998; Jensen et al., 1995b), and it is likely that a shift takes place towards the (more) reactive type of pig. Indeed, social structures seem to be more relaxed in domesticated pigs, and their aggression, flight distance and motility are lower than in wild pigs (Hemmer, 1990). The degree of variation in responses of the domesticated pig, though still being large, may be smaller than the original variation in responses of the wild ancestors. Hence, the degree of differentiation into coping strategies may have become weaker through domestication. A relatively large 'middle' group of animals emerges in which no clear strategies to deal with environmental changes can be detected. However, at the extremes, animals still tend towards different coping strategies, and they differ quantitatively and qualitatively in their behavioural and physiological response patterns to stress. These differences between population extremes seem to be large enough to influence susceptibility to stress and diseases.

Summarizing conclusions and practical implications

Mixing

From a welfare point of view, the best management procedure probably is to keep litter-mates together until slaughter, without disrupting social groups by mixing. This was also suggested in earlier research comparing two site systems, in which pigs are regrouped, with farrowing-to-finish systems, in which pigs are never mixed (Ekkel et al., 1995b; Scheepens et al., 1992). In The Netherlands, specific legislation (Varkensbesluit, 1998) within the frame of the Dutch Health and Welfare Act (Gezondheids- en Welzijnswet voor Dieren, 1992) has come into force, which allows unfamiliar pigs to be mixed only once, within one week after weaning. This measure will probably also be adopted by the European Commission, by amending existing EU legislation (EC Council Directive 91/630/EEC, 1991) on the protection of pigs to improve welfare conditions. The proposal of the Commission for amending Directive 91/630/EEC is based on a recommendation by its Scientific Veterinary Committee (1997). Despite these actions to improve the welfare of pigs, it should be emphasized that groupings imposed by farmers, although only done once, may cause long-term social instability and social stress, and hence reduced welfare. This thesis may provide different solutions to reduce mixing-associated welfare problems:

Hiding places and resources. In Chapter 4 we showed that when subordinate pigs are separated from dominants following initial fighting for dominance status (social defeat), they do not develop all signs of stress pathology normally seen after mixing. This possibly reflects a reduced state of stress of the subordinate by removal from the stressor, i.e. the dominant. It is therefore likely that in groups of pigs it is advantageous for subordinates to have possibilities to hide and not being confronted with dominants all the time, which means not having to compete for living and feeding space (Chapter 7; McGlone and Curtis, 1985; Rushen, 1987). As can be derived from Chapter 5, a provision of possibilities to decrease the number of interactions may also be beneficial for dominant pigs. The new legislation (Varkensbesluit, 1998) in The Netherlands, which is at present more restrictive than the European legislation (EC Council Directive 91/630/EEC, 1991), may to some extent meet these demands by the requirement that the minimal surface area per pig has to be increased (for pigs of 85-110 kg: from 0.7 m^2 to 1.0 m^2). However, one has to realize that the welfare of pigs cannot simply be translated into square meters per pig. An increase in feeding space (pigs tend to synchronize their feeding behaviour) and possibilities to hide and flee may additionally prove to be valuable measures to improve welfare of fattening pigs.

Subgroups of pigs. From our findings in Chapters 3 and 4, we conclude that the presence of familiar animals may have positive implications for welfare. We hypothesize that negative effects of mixing may be moderated by bringing subgroups of litter-mates together. Not only may familiar group members buffer against the adverse effects of (social) stress by processes of social support, the number and intensity of fightings may also be reduced through the presence of related conspecifics. When further experiments confirm this hypothesis, such a mixing routine can easily be implemented in modern pig farming.

Knowledge on coping strategies and aggression: the value of the backtest. Knowledge on specific coping strategies of individual pigs may be useful to improve welfare of pigs after mixing (Chapter 7). Bringing two proactive gilts together was most detrimental for welfare. In contrast, combining pigs with different coping strategies could lead to low levels of aggression and stress, but this strongly depended on the outcome of social fighting. Provision of variation in weight upon mixing was previously shown to reduce aggression after mixing (Andersen et al., 2000a; Rushen, 1987). Combined with our findings, the advantage for pig welfare by mixing pigs of different size may especially arise when the larger pigs are reactive and the smaller ones have proactive features. Nevertheless, it may not be easy or even impossible to implement this under commercial

conditions. In pig farming, sows are usually synchronized (one production group) to farrow simultaneously as much as possible, so that at the end of the fattening period, large numbers of growing pigs of similar weight can leave the farm at the same time to be slaughtered. Weight differences between pigs of the same production group may thus not be large enough, whereas differences in weight between pigs of different production groups become too large. Although the optimal social situation thus hardly can be established, the opposite, i.e. the worst social situation, may relatively easily be prevented. Mixing of many potentially aggressive pigs in one group can and should be avoided, based on our results of mixing two proactive pigs. For this purpose, the backtest is a valuable tool to be implemented by pig farmers. Routinely, piglets are handled several times at a young age and a (modification of a) backtest should therefore not require too much time and effort. As mentioned in Chapter 7, more research is needed to determine the type of relationships in groups becoming too large to form social hierarchies. When social relationships become weak, the backtest may have no practical value.

Individual housing

This thesis emphasizes that the pig is a socially living animal which requires social contact with conspecifics (Chapter 4). Individual housing of growing pigs is not routinely done, but individual housing of sows, in confinement systems such as stalls, is still common. In The Netherlands, national legislation prohibits sows without piglets to be housed individually (Varkensbesluit, 1998), and group-housing systems are therefore increasing. In line with the Dutch legislation, the European Commission will probably come with tougher regulations of individual housing of sows (amended EC Council Directive 91/630/EEC, 1991), by banning the use of individual pens for sows during a period starting from weaning to one week before the expected time of farrowing. By these new requirements, the living environment of sows may be much improved.

For experimental purposes, however, pigs are often individually housed, which facilitates experimental testing and controllability for investigators. However, the negative acute and long-term effects of this procedure may interfere with the outcome of experimental testings. Therefore, experimental work should as much as possible be done with grouped pigs, allowing interactions between members of this social species. Some experiments, however, require individual housing, e.g. for individual monitoring of pigs in metabolism cages. In this case, allowance of some contact with conspecifics may provide a solution to limit the negative effects of individual housing. The degree of exposure to conspecifics

seems to determine the level of stress caused by individual housing. In our experiments, individually housed pigs were not able to have visual and physical contact, which may be a high degree of social deprivation. The individually housed rats in Chapter 3 were able to see conspecifics through transparant walls, but this was not sufficient to recover from the detrimental effects of social defeat. However, it was recently shown that provision of (physical) contact through a wire mesh limits the negative effects of individual housing (Herskin and Jensen, 2000; Hurst et al., 1997). Thus, from a welfare point of view, separation, but not isolation, may provide the best procedure when individual housing of pigs is required.

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Samenvatting

Aanleiding en achtergrond van het onderzoek

Tal van factoren in de moderne varkenshouderij kunnen het welzijn en de gezondheid van de dieren negatief beïnvloeden. De moderne varkenshouderij is een zeer intensieve vorm van veehouderij waarin het welzijn van de dieren vaak ondergeschikt is aan het streven naar maximale bedrijfseconomische resultaten, leidend tot een grootschalige en efficiënte dierlijke productie. Dit soort houderij wordt ook wel met de naam bioindustrie aangeduid. Consumenten worden zich er steeds meer van bewust dat deze wijze van dierlijke productie ethisch niet verantwoord is. Deze bewustwording uit zich vooral in strengere regelgeving met als doel het welzijn van productiedieren te verbeteren. Echter, ondanks een verminderde maatschappelijke acceptatie van de bioindustrie, en een toenemende vraag naar 'welzijns-vriendelijke' producten, wordt het merendeel van de dieren nog steeds onder intensieve condities gehouden. Dit wordt vooral veroorzaakt door het aantrekkelijke prijsniveau van producten uit de bioindustrie.

De omstandigheden waarin het gedomesticeerde varken zich in de intensieve houderij bevindt wijken sterk af van de natuurlijke situatie. Hierdoor kunnen situaties ontstaan waarin de dieren niet de mogelijkheid hebben om soortspecifieke gedragingen uit te voeren. Dit kan leiden tot een sterk verminderde beheersbaarheid van omgevingsfactoren, waardoor stress kan ontstaan. Stress dient normaal als een effectief mechanisme voor aanpassing aan gebeurtenissen in de leefomgeving (adaptatie), en heeft als doel het interne evenwicht (homeostase) van het lichaam te waarborgen of te herstellen. Adaptatie komt tot stand door een nauw samenspel van veranderingen in gedrag, reacties van het centrale en autonome (sympathische en parasympathische) zenuwstelsel, en endocriene systemen. Een veroorzaker van stress, de stressor, is dus niet per definitie ongunstig. Evenzo is de reactie van het lichaam, oftewel de stressrespons, in het algemeen functioneel te noemen. Echter, in de moderne varkenshouderij komen condities voor waardoor zware eisen worden gesteld aan het adaptatievermogen (aanpassingsvermogen). Soms worden de grenzen van het adaptatievermogen zelfs overschreden. Dit kan leiden tot chronische stress en ziektes die optreden als gevolg van stress (stresspathologieën). Dit komt bijvoorbeeld voor als een bepaalde onbeheersbare situatie voor langere tijd aanhoudt, zoals sociale instabiliteit na mengen van vreemde (onbekende) dieren, slechte stalklimaatcondities, een hoge bezettingsgraad (weinig leefruimte per dier) en een kale, substraatarme leefomgeving. Wanneer een dier zich langdurig niet goed kan aanpassen, kan de chronische stressrespons op termijn schadelijk worden voor het individu en zijn sociale omgeving. Er kunnen abnormale gedragingen ontstaan zoals agressie,

gestoord gedrag (beschadigend gedrag zoals staartbijten; stereotyp gedrag) en apathie. Chronische stress kan ook leiden tot een hyperactiviteit van fysiologische regelsystemen. Langdurige activatie van het sympathisch zenuwstelsel, dat een stimulerende werking uitoefent op de frequentie van hartslag, met catecholamines (adrenaline en noradrenaline) als belangrijkste neurotransmitters, leidt bijvoorbeeld tot hart- en vaatafwijkingen. Het parasympathische zenuwstelsel heeft een onderdrukkend effect op hartslag en kan bij voortdurende activatie hartritme-stoornissen veroorzaken. De hypothalamus-hypofyse-bijnier-as reageert op stressoren door middel van afgifte van ACTH (adrenocorticotroop hormoon) uit de hypofyse en corticosteroïden uit de bijnierschors. Corticosteroïden, waaronder cortisol, hebben een belangrijke onderdrukkende werking op het immuunsysteem. Aanhoudende verhoging van cortisol kan daarom leiden tot een verminderd functioneren van het immuunsysteem en dus een verlaagde weerstand. Dit heeft tot gevolg dat ziekteverwekkers zoals bacteriën en virussen makkelijker een kans krijgen om dieren ziek te maken. Een verandering in fysiologische regelsystemen, met name in de hypothalamus-hypofyse-bijnier-as, kan ook optreden zonder dat basale niveaus van hormoonspiegels veranderen. Een dier kan dan bijvoorbeeld een meer dan normale stressrespons geven onder milde stresscondities. Dit kan duiden op een verhoogde gevoeligheid voor veranderingen in de omgeving, oftewel sensitisatie. Dit verschijnsel treedt bijvoorbeeld op na een éénmalige verlieservaring van ratten. Deze dieren ontwikkelen psychopathologieën welke sterke overeenkomsten vertonen met menselijke stress-gerelateerde stemmingsziektes, zoals angst en depressie. Een andere overeenkomst is een sterk gereduceerde beweeglijkheid en (sociale) activiteit. Over het optreden van veranderingen in emotionaliteit, leidend tot genoemde stemmingsziektes, is bij varkens, en landbouwhuisdieren in het algemeen, relatief weinig bekend.

Doel van het onderzoek

Van de factoren die het welzijn van vleesvarkens in de moderne houderij negatief kunnen beïnvloeden zijn vooral die factoren van belang die samenhangen met interacties tussen dieren, oftewel (psycho)sociale factoren. De sociale omstandigheden waaronder varkens worden gehouden in de intensieve houderij wijken dermate af van de natuurlijke situatie, dat de dieren minder of niet de mogelijkheid hebben hun 'normale' sociale gedrag uit te voeren. Als het dier hierdoor de controle over de omgeving verliest kan een toestand van stress en verminderd welzijn ontstaan. Wanneer stress ontstaat als gevolg van sociale factoren (sociale stressoren) spreken we ook wel van sociale stress. In de vrije

natuur leven varkens in een familieverband met een sterke sociale band tussen de dieren en verschillen in sociale status (rangorde). In de natuur worden rangordes geleidelijk gevormd wanneer de dieren opgroeien. Agressie in de vrije natuur blijft meestal beperkt omdat ondergeschikte dieren weg kunnen vluchten van dominante dieren.

In de intensieve houderij wordt gestreefd naar uniforme (in gewicht) groepen, en worden vleesvarkens uit verschillende tomen één of meerdere malen gemengd (in Nederland nu éénmalig toegestaan; Varkensbesluit). Onbekendheid met nieuwe hokgenoten en gelijk gewicht zijn nu juist die factoren die gevechten en agressie tussen dieren het meest in de hand werken. De gevechten die plaatsvinden om de rangorde vast te stellen gaan gepaard met veel sociale stress en verwondingen. Ook kan, in tegenstelling tot de situatie in de natuur, een rangorde niet geleidelijk gevormd worden. Ruimte en vluchtmogelijkheden zijn namelijk in zeer beperkte mate aanwezig in intensieve huisvestingssystemen, en vreemde dieren kunnen elkaar niet ontwijken. Hierdoor wordt ook het 'normale' sociale ritueel dat een verliezend (ondergeschikt) dier zich verwijdert van een dominant dier en ontwijkend gedrag gaat vertonen, ernstig belemmerd, en is het mogelijk dat agressie en stress gedurende een langere tijd aanhouden, leidend tot sociale instabiliteit. Op langere termijn kunnen zich problemen voordoen zoals gezondheidsproblemen en een verminderde groei. Laatstgenoemde problemen lijken vooral voor te komen bij ondergeschikte dieren. Mengen, onder de condities van de moderne houderij, is dus een belangrijke risicofactor voor het welzijn van vleesvarkens.

Het project dat in dit proefschrift wordt beschreven analyseert de gedragsmatige en fysiologische stressrespons van vleesvarkens tijdens het mengen van onbekende dieren, en bestudeert welke sociale componenten verantwoordelijk zijn voor de negatieve effecten van mengen: langdurig negatieve gevolgen van mengen kunnen voortkomen uit het aanhouden van stress doordat onbekende dieren zich niet aan elkaar kunnen onttrekken, maar zouden ook een lange-termijn effect kunnen zijn van gevechten om de rangorde te bepalen vlak na het mengen. Verder wordt in dit project aandacht besteed aan een tweetal kenmerken die het aanpassingsvermogen van individuele dieren kunnen beïnvloeden.

Social support. Bij verschillende diersoorten is bekend dat social support oftewel sociale ondersteuning door soortgenoten kan leiden tot een betere beheersbaarheid van omgevingsfactoren. Social support heeft hierdoor een stress-verlagende of stress-bufferende werking. Social support lijkt vooral op te kunnen treden tussen dieren met een sterke sociale band, zoals in relaties tussen moeder en

jong, maar ook tussen toomgenoten. Over het optreden van social support bij varkens is nauwelijks iets bekend. Een belangrijke doelstelling van dit project is om hier meer inzicht in te krijgen, vooral in samenhang met mengen. Door varkens sociaal te isoleren (individuele huisvesting) wordt vastgesteld of het hebben van sociaal contact op zichzelf belangrijk is voor het welzijn van de dieren.

Individuele dierkenmerken. Onderzocht wordt hoe individuele dierkenmerken het effect van mengen kunnen beïnvloeden en welke rol deze kenmerken spelen in het vermogen en de wijze van aanpassing aan omgevingsveranderingen (coping). Het bestaan van verschillende types, overeenkomend met reactieve (voorheen passief genoemd) en proactieve (voorheen actief genoemd) laboratoriumratten en -muizen (genetische lijnen) en dieren in wilde populaties huismuizen en koolmezen (tweedeling in de populatie), zal nader bestudeerd worden. Proactieve dieren worden gekenmerkt door gedrag dat lijkt op 'eerst doen, dan denken'. Ze vormen snel routines en zijn relatief agressief van aard. Fysiologisch gezien hebben ze een uitgesproken reactie van het sympathische zenuwstelsel. Reactieve dieren zijn afwachtender en hun reacties op stressoren lijken juist op 'eerst denken, dan doen'. Ze lijken flexibeler met de omgeving om te kunnen gaan en zijn daardoor in staat zich makkelijker aan te passen aan veranderende omgevingscondities. Indien een reactie vereist is wordt hun fysiologie meer gekenmerkt door een relatief hoge activiteit van de hypothalamus-hypofyse-bijnier-as en het parasympathische zenuwstelsel. Genoemde verschillen geven aan dat het hebben van een bepaalde copingstrategie de perceptie van een situatie, de dynamiek van adaptatiemechanismen, en het type stresspathologie in belangrijke mate beïnvloedt. Een keuze voor een bepaalde copingstrategie wordt voornamelijk gestuurd door erfelijke factoren, maar kan door ervaring worden beïnvloed. De onzekerheid omtrent het bestaan van copingstrategieën bij gedomesticeerde varkens komt voort uit tegenstrijdige conclusies uit voorgaand onderzoek. Tevens wordt aangenomen dat domesticatie een belangrijke selectiedruk op varkens uitgeoefend heeft. Het is daarom niet ondenkbaar dat een verschuiving is opgetreden binnen de populatie naar een bepaald type dat zich beter aanpast aan opgelegde condities.

Hoewel voor het onderzoek in het onderhavige project een hoofdzakelijk fundamentele benadering is gekozen, zullen aanbevelingen en praktische handvaten worden aangedragen ter verbetering van het welzijn van vleesvarkens binnen het kader van de moderne varkenshouderij. Om welzijnsproblemen op een verantwoorde en objectieve wijze te identificeren hebben we in dit project gekozen

voor een multidisciplinaire aanpak door een integratie van (sub)disciplines zoals ethologie (gedragsstudies), fysiologie, immunologie en endocrinologie.

Resultaten onderzoek

Hoofdstuk 2

Het project start met een studie naar de dag-nacht kinetiek (24-uurs kinetiek of circadiane ritmiek) van cortisol in speeksel van groeiende vleesvarkens, met als doel dit hormoon als belangrijke en makkelijk meetbare indicator voor stress te kunnen gebruiken. Cortisol is één van de meest gebruikte fysiologische indicatoren van stress. Een belangrijk voordeel van metingen van cortisol in speeksel is dat speekselmonsters op een relatief eenvoudige en 'stressvrije' manier af te nemen zijn. Dit is belangrijk in onderzoek waarbij effecten van behandelingen op stressparameters wordt onderzocht. Meetmethoden dienen namelijk zelf zo weinig mogelijk stress te veroorzaken. Het alternatief voor het afnemen van speeksel, herhaaldelijke bloedafname door middel van een catheter die operatief moet worden ingebracht of door punctie van de halsader, geeft vele malen meer ongerief, waardoor stress kan optreden. Een ander belangrijk voordeel van metingen aan cortisol in speeksel is dat dit hormoon in speeksel een afspiegeling is van de vrije (niet gebonden aan drager-eiwitten) en biologisch actieve fractie in plasma. Van cortisol, gemeten in bloedplasma, is bekend dat dag-nacht fluctuaties voorkomen. Tevens varieert de gevoeligheid van het systeem waarvan cortisol deel uitmaakt (hypothalamus-hypofyse-bijnier-as) gedurende de dag. Dit bemoeilijkt de interpretatie van cortisolniveaus gedurende experimentele settings, en dan met name veranderingen in niveaus en vergelijkingen tussen periodes en/of behandelingen.

Uit het experiment blijkt dat leeftijd een belangrijk effect heeft op het ritme van cortisol in speeksel. In de leeftijdsperiode van 12 tot 24 weken daalt de gemiddelde concentratie van dit hormoon en ontwikkelt zich een ritme dat omstreeks de leeftijd van 20 weken een volwassen en stabiel profiel bereikt. Blootstelling aan een stressor (isolatie) gedurende twee perioden van de dag, 's ochtends en 's avonds, en op twee leeftijden (12 en 20 weken), laat zien dat de cortisol respons 's ochtends hoger is, wanneer ook het basale niveau het hoogst is. Borgen hebben gemiddeld gezien hogere cortisolniveaus dan gelten. Met het voorlaatste gegeven wordt verder in dit project zoveel mogelijk rekening gehouden door (de aanvang van) blootstelling aan stressoren en testen te beperken tot een bepaald dagdeel, met name de ochtend, wat de interpretatie van gemeten waardes aanzienlijk vergemakkelijkt. Voor de bepaling van cortisol in speeksel van varkens

is tijdens dit experiment een radioimmunoassay (RIA) geoptimaliseerd en gevalideerd.

Hoofdstukken 3 en 4

Het is niet bekend of de langdurige negatieve effecten van mengen van vleesvarkens veroorzaakt worden door de sociale stress die gepaard gaat met initiële hevige gevechten en agressie om de rangorde te bewerkstelligen en/of door het aanhouden van stress doordat onbekende dieren zich niet voor elkaar kunnen verschuilen. Om hier meer inzicht in te krijgen wordt allereerst onderzocht wat de gevolgen zijn van verlies tijdens een sociale confrontatie (sociaal verlies). De verwachting is dat de meeste stress optreedt bij verliezende dieren, omdat met name voor deze dieren een verlaagde beheersbaarheid van de omgeving ontstaat. In eerste instantie wordt gebruik gemaakt van een model wat toegepast wordt in het biomedisch onderzoek en is gebaseerd op een éénmalig sociaal verlies van ratten (*Hoofdstuk 3*). Bij mannelijke ratten is bekend dat een acuut sociaal verlies een traumatische ervaring is en langdurige negatieve gevolgen voor het verliezende dier heeft. Sociaal verlies lijkt zelfs te leiden tot verschijnselen die overeenkomen met menselijke stemmingsziektes zoals angst en depressie. Echter, het rattenmodel maakt gebruik van dieren die na een verlies individueel gehuisvest zijn. De rat is een sociaal dier en leeft onder natuurlijke condities in familieverband, net als het varken. Daarom wordt in *Hoofdstuk 3* onderzocht of dieren baat hebben bij de aanwezigheid van bekende soortgenoten (social support) na een verlieservaring. Het blijkt dat dit inderdaad het geval is. De stresservaring is op langere termijn veel beter verwerkt door dieren die met bekende soortgenoten zijn gehuisvest, dan door dieren die in hun eentje het verlies moeten verwerken. Karakteristieken van laatstgenoemde dieren, overeenkomend met symptomen van depressiviteit bij mensen, zijn o.a. een lage activiteit, groei (gewichts-) afname, en een hoge activiteit van de hypothalamus-hypofyse-bijnier-as.

In *Hoofstuk 4* wordt uitgaande van het verliesmodel bij ratten onderzocht in hoeverre 10 weken oude varkens (gelten) op korte en langere termijn (gedurende 3 weken) negatieve gevolgen van een verlieservaring ondervinden, met hun sociale omgeving als modulerende factor. Daartoe wordt, net als bij het rattenmodel, gebruik gemaakt van de 'resident-intruder' test. Na een kortdurende confrontatie met een dominant varken wordt een verliezend varken hetzij teruggezet bij een bekende soortgenoot (toomgenoot) hetzij individueel gehuisvest. De acute fysiologische reactie, vooral gekenmerkt door hoge activiteit van de hypothalamus-hypofyse-bijnier-as en het sympathische zenuwstelsel, maar ook door

verschuivingen in percentages van bepaalde witte bloedcelpopulaties (leucocyten: daling in %lymfocyten, stijging in %neutrofyle granulocyten), geeft aan dat sociaal verlies een sterke stressor is. De acute stressrespons verdwijnt echter sneller en varkens zijn beter in staat te habitueren aan een herhaalde angstopwekkende test (vreemde omgeving), met andere woorden er treedt sneller gewenning op, wanneer ze na verlies bij een toomgenoot worden teruggeplaatst. Social support lijkt dus ook bij varkens op te treden en heeft een gunstig effect op het aanpassingsvermogen van de dieren. Echter, varkens met een verlieservaring hebben op langere termijn toch altijd nog nadeel van de stresservaring, ongeacht de sociale omgeving. Ze zijn en blijven verhoogd gevoelig, oftewel ze zijn gesensitiseerd, voor veranderingen in hun omgeving (bijv. introductie van een vreemd voorwerp, confrontatie met een voorheen aversieve stimulus). Dit uit zich vooral in een hoge hartslag in genoemde testsituaties. Hetzelfde geldt voor dieren die individueel zijn gehuisvest. Aanvankelijk heeft sociale isolatie geen nadelige consequenties voor laatstgenoemde dieren, zoals waargenomen bij controledieren die geen sociaal verlies hebben ondergaan. Echter, op langere termijn gaan sociaal geïsoleerde dieren, overeenkomend met dieren met een verlieservaring, minder goed om met acute (milde) stressoren. Herhaalde plaatsing in een vreemde omgeving geeft geen habituatie te zien (onveranderd hoge locomotie en cortisol respons). Ook hebben deze dieren een extreem hoge hartslag in diverse testsituaties.

Concluderend kan gezegd worden dat de vaak beschreven negatieve effecten van mengen niet alleen toegeschreven kunnen worden aan stress als gevolg van initiële gevechten, leidend tot dominante en ondergeschikte dieren. Verliezende varkens vertonen wel een aantal kenmerken van langdurig negatieve gevolgen na de verlieservaring, maar hun groei is bijvoorbeeld niet verminderd. Vaak is juist een verminderde groei een belangrijke negatieve consequentie van mengen. Dit wijst erop dat de risico's van mengen voor het dierenwelzijn gelegen zijn in de permanente confrontatie tussen vreemde dieren. Een andere belangrijke conclusie is dat sociaal contact belangrijk is voor een varken, wat blijkt uit een aangetast welzijn bij sociaal geïsoleerde dieren.

Hoofdstuk 5

In het experiment beschreven in *Hoofdstuk 5* wordt onderzocht wat het effect van mengen is op de afweer tegen ziektes. Daarvoor wordt een vaccinatie-challenge model gebruikt, met het pseudorabies virus (PRV; veroorzaker van de ziekte van Aujeszky) als ziekteverwekker. Geslacht en sociale status worden in dit

experiment als proeffactoren meegenomen. Na het mengen van specifiek-pathogeen-vrije 6-7 weken oude gelten en borgen (paarsgewijs, hetzelfde geslacht) blijkt dat mengen negatieve gevolgen kan hebben op de immuunrespons en de verkregen bescherming tegen het virus. Dit is vooral zichtbaar bij borgen die onderling gemengd zijn. De intensiteit en niveaus van agressie, en catecholamineniveaus, verschilden niet tussen gemengde gelten en gemengde borgen. Wel zijn er aanwijzingen voor hogere cortisolniveaus na mengen in borgen, wat eerder ook in *Hoofdstuk 2* werd aangetoond voor basale condities. Dit verschil in hypothalamus-hypofyse-bijnier-as activiteit tussen borgen en gelten geeft aanleiding tot discussie over de effecten van castratie. Een verhoogde hypofyse-bijnier-as-activiteit heeft een verlaging van weerstand tegen ziektes tot gevolg, en kan een lange-termijn effect (sensitisatie) zijn van castratie. Het kan ook een direct gevolg zijn van deze pijnlijke ingreep (verlaging van testosteron spiegels). In dit experiment hebben de dominante dieren in zijn algemeenheid een betere immuunrespons dan ondergeschikte dieren, maar na mengen lijken ze in sommige opzichten juist over een lagere immuuncompetentie te beschikken dan gemengde ondergeschikte dieren. Dit is misschien een onverwacht resultaat, maar het benadrukt dat de sociale situatie die ontstaat na mengen ook nadelig is voor dominante dieren. Deze dieren ervaren een continue dreiging hun dominante status te verliezen door de permanente nabijheid van andere dieren.

Hoofdstukken 6-8

In hoofdstukken 6-8 wordt onderzocht hoe individuele varkens (gelten) verschillen in het aanpassingsvermogen aan gebeurtenissen in de omgeving. In *Hoofdstuk 6* wordt allereerst getracht het bestaan van verschillende types aan te tonen, oftewel te bewijzen dat de varkenspopulatie onderverdeeld kan worden in dieren met een reactieve dan wel met een proactieve copingstrategie. Metingen aan agressie in een herhaalde voercompetitietest laten zien dat dit een stabiel dierkenmerk is. Een rugtest, uitgevoerd enkele dagen na geboorte, lijkt voor een gedeelte van de populatie een voorspellende waarde voor latere agressie en manier van coping te hebben. In de rugtest wordt een big op de rug gelegd en gedurende één minuut in deze houding vastgehouden. Doet een big veel pogingen om te ontsnappen dan heeft het dier later een relatief agressief karakter en heeft het een proactieve(re) manier van omgaan met stress. Weinig weerstand in de rugtest geeft aan dat het dier voornamelijk rustig en reactiever van aard is. Voor ongeveer de helft van de dieren (middengroep) is geen voorkeur voor een bepaalde copingstrategie aan te geven. Een tweedeling in de populatie kan dus niet worden

aangetoond, wat een belangrijke conclusie is in dit onderzoek. Een nadere bestudering van de extreem reagerende biggen laat zien dat gedurende de zoogperiode het vooral de proactieve biggen zijn die de voorste en beste tepels bezetten en het hardst groeien. Zoals blijkt uit de reactie op 'vreemde stimuli' vertonen proactieve dieren kortere latentietijden en lijken ze minder geremd te worden in hun gedrag. Reactieve dieren lijken meer exploratiegedrag te vertonen als reactie op 'vreemde stimuli', en hebben fysiologisch gezien een hogere hypothalamus-hypofyse-bijnier-as reactiviteit. Dit laatste wordt ook duidelijk na maximale stimulatie van de bijnieren door middel van het toedienen van ACTH.

In *Hoofdstuk 7* wordt vervolgens getracht de oorzaken van agressie en stress na mengen te herleiden naar de eigenschappen van individuele dieren. Om hier greep op te krijgen worden alleen dieren met een voorkeur voor een bepaalde copingstrategie experimenteel ingezet. Na identificatie van reactieve(re) en proactieve(re) gelten aan de hand van de rugtest worden deze dieren op een leeftijd van 7 weken paarsgewijs gemengd in verschillende combinaties. Ten opzichte van de andere combinaties geeft het mengen van twee proactieve dieren een minder snelle afname van agressie te zien, wat zich onder andere ook uit in relatief veel huidbeschadigingen bij deze paren gedurende de eerste week na mengen. Relatief hoge niveaus aan catecholamines (in urine) en een hoge lichaamstemperatuur op de eerste dag, en een mindere voorefficiëntie en hogere basale hypothalamus-hypofyse-bijnier-as activiteit (ACTH) gedurende de eerste week, geven aan dat binnen deze paren van alleen proactieve gelten ook de meeste stress optreedt. Sociale 'frictie' of sociale instabiliteit komt dus vooral voor wanneer proactieve dieren gemengd worden. Opvallend is echter dat sociale instabiliteit ook voor kan komen bij paren bestaande uit verschillende 'types'. Dit blijkt te gebeuren wanneer een proactief dier dominant wordt, wat in ongeveer de helft van de gevallen gebeurt. In tegenstelling tot een reactief dier, is een proactief dier na het winnen van een gevecht minder geremd in uitingen van agressie. Dominantie van een reactief dier, wat zelf alleen agressief is gedurende het vechten om de dominante positie, lijkt echter de agressie van een proactief dier te onderdrukken. Het samenbrengen van verschillende 'types' kan dus zowel gunstig als ongunstig uitpakken. Paren bestaande uit alleen reactieve dieren zijn wat hun kenmerken en reacties betreft een 'midden-groep'. Uit deze combinatie komt niet de meest gunstige, maar ook niet de meest ongunstige, sociale situatie voort.

Ondanks de genoemde grote verschillen tussen en binnen de combinaties blijkt dat ondergeschikte dieren in het algemeen meer nadelige effecten ondervinden van de mengervaring dan dominante dieren. Ondergeschikte dieren hebben acuut gezien,

dus als direct gevolg van sociaal verlies, sterkere stijgingen in speeksel-cortisolniveaus en lichaamstemperatuur, en ze vocaliseren meer. Hun reactie op een angst-inducerend 'vreemd voorwerp' (één week na mengen) wordt gekenmerkt door een hogere frequentie van vocaliseren en een hoge parasympathische respons. Dit laatste dient waarschijnlijk als compensatie voor een verhoogde sympathische activiteit: de gemiddelde hartslag verschilde niet tussen dominante en ondergeschikte dieren. De bevinding dat ondergeschikte dieren achterblijven in groei bij dominante dieren bevestigt de conclusies van *Hoofdstuk 4* dat de risico's van mengen gelegen zijn in de permanente confrontaties tussen dominante en ondergeschikte dieren. Het ondergeschikt zijn in een groep met vreemde dieren heeft waarschijnlijk meer stress tot gevolg dan het ondergeschikt zijn in een groep met bekende soortgenoten. De beschreven lange-termijn gevolgen van een sociaal verlies (*Hoofdstuk 4*) en bovengenoemde kenmerken van ondergeschikte gelten na mengen kunnen een aanwijzing zijn voor het optreden van stemmingsstoornissen zoals angst en depressie bij varkens die gemengd worden onder intensieve houderijcondities.

Hoofdstuk 8, tenslotte, behandelt de reacties van reactieve(re) en proactieve(re) 7 weken oude gelten op sociale isolatie gedurende 3 weken. In tegenstelling tot het experiment in *Hoofdstuk 4*, worden dieren ten behoeve van isolatie verplaatst naar een ander hok (zie ook *Hoofdstuk 2*). Ook al is deze procedure stressvol voor beide 'types', toch zijn er duidelijke verschillen in stressreacties. Reactieve gelten hebben direct na isolatie een hogere stijging in het niveau van speekselcortisol en vertonen in het algemeen meer exploratiegedrag. Proactieve dieren zijn in het eerste uur na isolatie onrustiger (veel lopen en vocaliseren) en hebben een hogere uitscheiding van noradrenaline in de urine na 3 weken. Deze reactiepatronen komen overeen met verwachte reacties op basis van copingstrategieën. Echter, de meeste reacties op langere termijn geven vooral aan dat sociale isolatie meer problemen geeft voor het proactieve 'type'. Lichaamstemperatuur, na een eerdere daling, keert in proactieve gelten niet en in reactieve gelten wel (binnen 1 dag) terug op het niveau van vóór isolatie. Parasympathische activiteit, normaal hoger in reactieve dieren, is na 1 week isolatie juist hoger in proactieve dieren (tijdens confrontatie met een 'vreemd voorwerp'), waarschijnlijk ter compensatie van sympathische activiteit. Verschuivingen in percentages van eerder genoemde leucocytenpopulaties treden alleen bij proactieve dieren op. Eén proactieve gelt ontwikkelde maagzweren. Sociale isolatie heeft dus voor proactieve dieren meer het karakter van een chronische stressor dan voor reactieve dieren. In hoeverre dit doorgetrokken kan

worden naar andere omgevingsveranderingen is de vraag, maar het kan een aanwijzing zijn voor een flexibeler karakter van reactieve varkens. Theoretisch zouden laatstgenoemde varkens zich dan beter kunnen handhaven in de intensieve houderij. Dit kan een verklaring zijn voor een verdwenen 'tweedeling' in de populatie door een selectiedruk (domesticatie) richting een reactieve(re) manier van coping. Echter, de extremen verschillen (nog) zodanig in de manier van aanpassingsmechanismen dat dit relevant is voor de relatie tot de omgeving en het optreden van stresspathologieën.

Conclusies en aanbevelingen

Het project dat in dit proefschrift wordt beschreven laat zien dat in de sociale omgeving van intensief gehouden vleesvarkens niet alleen negatieve (sociaal verlies, sociale instabiliteit) maar ook positieve componenten (social support) een belangrijke rol spelen. De balans tussen deze componenten heeft een belangrijke invloed op het niveau van dierenwelzijn, en inzicht hierin biedt mogelijkheden om het welzijn van in groepen gehouden vleesvarkens te verbeteren door adequate veranderingen in management en huisvesting. De belangrijkste conclusies van dit project zijn:

- De aanwezigheid van bekende soortgenoten heeft een positief effect op het welzijn van vleesvarkens. Bekende soortgenoten (toomgenoten) zijn in staat social support (sociale ondersteuning) te bieden aan dieren met een stresservaring, zoals bij de verwerking van een sociaal verlies. Verondersteld wordt daarom dat de negatieve effecten van mengen beperkt kunnen worden als dieren zoveel mogelijk met bekende soortgenoten in een nieuwe groep terechtkomen. Niet alleen kunnen het aantal en de intensiteit van gevechten om de rangorde daardoor afnemen, het contact met bekende soortgenoten kan door het optreden van social support tevens een stressverlagende of stressbufferende werking hebben.
- Alhoewel mengen ook nadelig is voor het welzijn van dominante dieren, zijn het vooral de ondergeschikte dieren die het meest te lijden hebben onder deze routinehandeling. De nadelige effecten van mengen zijn toe te schrijven aan de abrupte wijze waarop onbekende dieren in een nieuwe groep een rangorde moeten vormen en niet de kans hebben zich aan elkaar te onttrekken. Dit wordt vooral veroorzaakt door de minimale leefruimte en de afwezigheid van vluchtmogelijkheden. Agressie en sociale stress kunnen daardoor gedurende een lange tijd aanhouden en leiden tot sociale instabiliteit.

- Varkens zijn sociale dieren waarvoor het hebben van sociaal contact een belangrijke levensbehoefte is. Wanneer sociaal contact onmogelijk wordt gemaakt leidt dit tot een verminderd welzijn.
- De varkenspopulatie kan niet onderverdeeld worden in dieren met een reactieve dan wel met een proactieve copingstrategie (geen tweedeling). Dit zou verband kunnen houden met domesticatie waardoor een selectiedruk richting een bepaalde type dier kan hebben plaatsgevonden. Het reactieve type lijkt hier het meest voor in aanmerking te komen. Echter, voor 'extremen' geldt nog steeds dat ze een voorkeur hebben of neigen naar een bepaalde manier van coping. Met een rugtest op zeer jonge leeftijd is een voorspelling te doen van de manier van coping en het daarbij behorende agressieniveau. Het mengen van dieren met een groot verschil in agressieve intenties kan leiden tot sociaal zeer stabiele groepen, maar dat is sterk afhankelijk van de uiteindelijke rangorde. Voor de praktijk is het daarom van belang in ieder geval de agressievere (proactieve(re)) dieren zo goed mogelijk over groepen te verspreiden, waardoor wordt voorkomen dat zeer instabiele sociale groepen met veel gezondheidsproblemen en een lage productie ontstaan. In de praktijk zou de rugtest dus gebruikt kunnen worden voor een betere groepssamenstelling bij het mengen van vleesvarkens. Dit zou ook kunnen gelden voor in groepen gehouden zeugen.

List of publications

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Dankwoord

Het begon allemaal in 1994. In dat jaar kreeg ik de kans om na een paar jaar 'andere dingen doen' de draad van het doen van wetenschappelijk onderzoek weer op te pakken. Tijdens mijn studie Biologie aan de Landbouwuniversiteit Wageningen had ik me al in menig afstudeervak en stage toegelegd op de gevolgen van stress voor mens en dier. Mijn eerste aanraking met varkens kwam echter pas in genoemd jaar. Op het toenmalig Instituut voor Veeteeltkundig Onderzoek (IVO-DLO) te Zeist wilde men een start maken met de ontwikkeling van een stressmodel bij varkens om de relatie tussen stress en weerstand tegen ziektes te bestuderen. Daar had ik wel oren naar. Harry Blokhuis was degene die mij toen de gelegenheid gaf om hieraan te gaan werken. Bedankt Harry.

Begin 1995 staken een aantal onderzoeksinstituten in Nederland de koppen bij elkaar om te komen tot een voorstel voor een onderzoeksprogramma met als titel 'Determinanten van adaptief vermogen'. Een belangrijk aspect van dit programma was het vergroten van het fundamentele inzicht in de processen die de kwetsbaarheid van individuele dieren vergroten of verkleinen. Het programma was gericht op de intensieve houderij, en het varken werd gekozen als belangrijkste proefdier. Door het IVO, om precies te zijn door Joop te Brake, Harry Blokhuis en mijn persoon, is toen een deelproject uitgewerkt, met de titel: 'Meervoudige stressoren en coping style: effect van stress op weerstand tegen ziektes bij varkens'. Toen de financiering werd toegekend kwam ik in de gelukkige omstandigheid om het project te mogen gaan uitvoeren. Daarmee was het startsein gegeven voor het werken aan mijn promotie. De officiële start van het project kwam begin 1996, omstreeks de verhuizing van het IVO naar Lelystad (fusie met het CDI: Centraal Diergeneeskundig Instituut), waardoor het huidige Instituut voor Dierhouderij en Diergezondheid (ID-Lelystad, gemakshalve 'het ID') ontstond. Lelystad werd mijn werkplek, maar als promovendus werd ik verbonden aan de Rijksuniversiteit Groningen. Dat was het begin van een meer dan prettige samenwerking met mijn promotor Jaap Koolhaas. Van Jaap heb ik bijzonder veel geleerd, met name op het gebied van wetenschappelijk inzicht en logisch redeneren. Jaap, jouw kritische en stimulerende begeleiding, samen met die van Harry, liet weinig te wensen over. Ik had ook een prima medium om met jou, Harry, en verschillende andere collega's van het ID over de diepgang en voortgang van het onderzoek te kunnen praten: ons AIO-overleg. Dit overleg was met name interessant omdat hier ook de promotie-projecten van collega-AIO's Ingrid de Jong en Johanna de Groot in besproken werden. Ingrid en Johanna zijn daarom nauw betrokken geweest bij mijn onderzoek, en onze projecten waren dan ook perfect op elkaar afgestemd. Ingrid en Johanna, bedankt voor de zeer plezierige samenwerking. De afstemming op het

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Marko Ruis werd geboren op 29 mei 1965 te Breda. Na het doorlopen van de MAVO (Witrijt, Rijen, diploma 1981) en de HAVO (Gerardus Majella, Dongen, diploma 1983), behaalde hij in 1985 het VWO diploma aan het Schaepmancollege te Dongen. In datzelfde jaar begon hij met de studie Biologie aan de Landbouwuniversiteit Wageningen (LUW). In de afstudeerfase lag het accent op *ethologie* (vakgroep Veehouderij, LUW: 'Het optreden van stereotypieën bij wolven in gevangenschap", locatie: Ouwehands Dierenpark, Rhenen*), stress-fysiologie* (vakgroep Fysiologie van Mens en Dier, LUW: 'De rol van neuropeptiden van het stress-systeem in de regulatie van LH afgifte in de rat'; en Department of Zoology, Oregon State University, Corvallis, Oregon, USA: 'Effects of acute stress on blood clotting and yeast killing by phagocytes of rainbow trout'), en *immunologie* (vakgroep Experimentele Diermorfologie en Celbiologie, LUW: 'De systemische en/of mucosale immuunrespons in de karper na orale, anale of intramusculaire vaccinatie met Vibrio anguillarum bacterin'; en Afdeling Immunologie, Wilhelmina Kinderziekenhuis, Universiteit Utrecht: 'Inhibition of tyrosine phosphorylation potentiates ß-adrenergic receptor-mediated signal transduction'). In augustus 1991 studeerde hij af.

Na het volgen van een post-academische opleiding Milieumanagement (CBBM/Elsevier, diploma 1993), trad hij in januari 1994 in dienst als onderzoeksmedewerker bij het voormalig Instituut voor Veeteeltkundig Onderzoek (IVO-DLO) te Zeist. Van februari 1996 tot juli 2000 werkte hij als Assistent in Opleiding (AIO) bij de vakgroep Dierfysiologie van de Rijksuniversiteit Groningen. Dit proefschrift is daarvan het resultaat. Het onderzoek werd grotendeels uitgevoerd bij de afdeling Gedrag, Stressfysiologie en Management van het Instituut voor Dierhouderij en Diergezondheid te Lelystad (ID-Lelystad).

Marko Ruis is sinds september 2000 werkzaam als wetenschappelijk onderzoeker welzijn van legpluimvee en konijnen bij de divisie Pluimvee, Nertsen en Konijnen van het Praktijkonderzoek Veehouderij (PV) in Lelystad:

Praktijkonderzoek Veehouderij
Postbus 2176
8203 AD Lelystad
m.a.w.ruis@pv.agro.nl

Research Institute for Animal Husbandry
P.O. Box 2176
8203 AD Lelystad, The Netherlands